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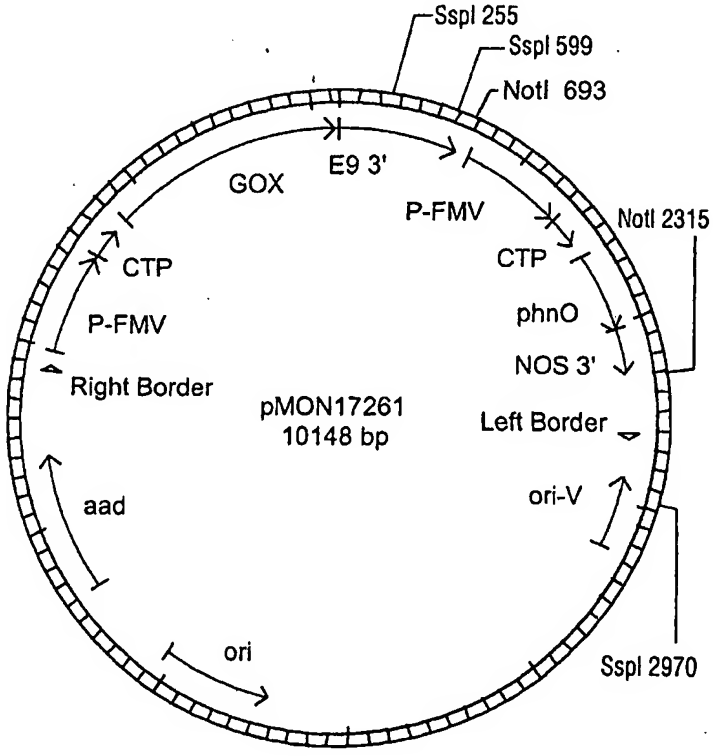
(57) Abstract

The invention relates in general to herbicide resistance in plants, and more particularly to a new class of phosphonate metabolizing genes, and methods of using these genes for improving plant tolerance to phosphonate herbicides.



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PHOSPHONATE METABOLIZING PLANTS

Reference to Prior Applications

This application claims the benefit of priority to US Provisional Application Serial No. 60/108,763 filed November 17, 1998.

Field of the Invention

The present invention relates in general to herbicide resistance in plants, and more particularly to a new class of phosphonate metabolizing genes and methods of using these genes for improving plant tolerance to phosphonate herbicides.

Description of the Prior Art

Phosphorous containing organic molecules can be naturally occurring or synthetically derived. Organic molecules containing phosphorous-carbon (C-P) bonds are also found naturally or as synthetic compounds, and are often not rapidly degraded, if at all, by natural enzymatic pathways. Synthetic organophosphonates and phosphinates, compounds that contain a direct carbon-phosphorous (C-P) bond in place of the better known carbon-oxygen-phosphorous linkage of phosphate esters (Metcalf et al., Gene 129:27-32, 1993), have thus been widely used as insecticides, antibiotics, and as herbicides (Chen et al., J. Biol. Chem. 265:4461-4471, 1990; Hilderbrand et al., *The role of phosphonates in living systems*, Hilderbrand, R.L., ed, pp. 5-29, CRC Press, Inc., Boca Raton, FL, 1983). Phosphonates are ubiquitous in nature, and are found alone and in a diversity of macromolecular structures in a variety of organisms (Jiang et al., J. Bacteriol. 177:6411-6421, 1995). Degradation of phosphonate molecules proceeds through a number of known routes, a C-P lyase pathway, a phosphonatase pathway, and a C-N hydrolysis pathway (Wanner, Biodegradation 5:175-184, 1994; Barry et al., US Patent No. 5,463,175, 1995). Bacterial isolates capable of carrying out these steps have been characterized (Shinabarger et al., J. Bacteriol. 168:702-707, 1986; Kishore et al., J. Biol. Chem. 262:12,164-12,168, 1987; Pipke et al., Appl. Environ. Microbiol. 54:1293-1296,1987; Jacob et al., Appl. Environ. Microbiol. 54:2953-2958, 1988; Lee et al., J. Bacteriol. 174:2501-2510, 1992; Dumora et al., Biochim. Biophys. Acta 997:193-198, 1989; Lacoste et al., J. Gen. Microbiol. 138:1283-1287, 1992). However, with the exception of phosphonatase and glyphosate oxidase (GOX), other enzymes capable of carrying out these reactions have not been characterized.

Several studies have focused on the identification of genes required for C-P lyase degradation of phosphonates. Wackett et al. (J. Bacteriol. 169:710-717, 1987) disclosed broad substrate specificity toward phosphonate degradation by *Agrobacterium radiobacter* and specific utilization of glyphosate as a sole phosphate source. Shinabarger et al. and Kishore et al. disclosed C-P lyase degradation of the phosphonate herbicide, glyphosate, to glycine and inorganic phosphate through a sarcosine intermediate by *Pseudomonas* species.

E. coli B strains had previously been shown to be capable of phosphonate utilization (Chen et al.), whereas *E. coli* K-12 strains were incapable of phosphonate degradation. However, K-12 strains were subsequently shown to contain a complete, though cryptic, set of genes (*psi*D or *phn*) capable of phosphonate utilization (Makino et al.), as mutants were easily selected by growth on low phosphate media containing methyl- or ethyl-phosphonate as sole phosphorous sources. Such K-12 strains adapted for growth on methyl- or ethylphosphonate were subsequently shown to be able to utilize other phosphonates as sole phosphorous sources (Wackett et al., J. Bacteriol. 169:1753-1756, 1987).

Avila et al. (J. Am. Chem. Soc. 109:6758-6764, 1987) were interested in the mechanistic appraisal of biodegradative and detoxifying processes as related to aminomethyl-phosphonates, including elucidating the intermediates, products, and mechanisms of the degradative dephosphorylation process. Avila et al. studied the formation of dephosphorylated biodegradation products from a variety of aminophosphonate substrates in *E. coli* K-12 cultures previously adapted to growth on ethylphosphonate. Furthermore, Avila et al. utilized N-acetyl-AMPA (N-acetyl-amino-methyl-phosphonate) as a sole phosphate source in some of their studies in order to show that acetylated AMPA was not inhibitory to C-P bond cleavage. In addition, Avila et al. noted that N-acetyl-AMPA was able to serve as a sole phosphate source during *E. coli* K-12 growth, however, they did not observe N-acetyl-AMPA formation when AMPA was used as a sole phosphate source. Their results indicated that AMPA was not a substrate for acetylation in *E. coli*.

Chen et al. identified a functional *psi*D locus from *E. coli* B by complementation cloning into an *E. coli* K-12 strain deficient for phosphonate utilization, which enabled the K-12 strain to utilize phosphonate as a sole phosphate source (J. Biol. Chem. 265:4461-4471, 1990). Chen et al. thus disclosed the DNA sequence of the *psi*D complementing locus, identified on a 15.5 kb *Bam*HI fragment containing 17 open reading frames designated *phn*A-*phn*Q, comprising the *E.*

coli B *phn* operon. The cryptic *phn* (*psi*D) operon from *E. coli* K-12 was subsequently found to contain an 8-base pair insertion in *phn*E. The resulting frameshift in *phn*E not only results in defective *phn*E gene product, but also apparently causes polar effects on the expression of downstream genes within the operon, which prevent phosphonate utilization (Makino et al., J. Bacteriol. 173:2665-2672, 1991). The operon has been more accurately described to contain the genes *phn*C-*phn*P by the work of Makino et al. Further research has been directed to understanding the nature of the function of each of the genes within this operon (Chen et al., J. Biol. Chem. 265:4461-4471, 1990; Makino et al., J. Bacteriol. 173:2665-2672, 1991; Wanner et al., FEMS Microbiol. Lett. 100:133-140, 1992; Metcalf et al., Gene 129:27-32, 1993; Ohtaki et al., Actinomyceteol. 8:66-68, 1994). In all of these efforts, the *phn*O gene has been implicated as a regulatory protein based on its similarity to other nucleotide binding proteins containing structural helix-turn-helix motifs. Furthermore, mutagenesis of genes in the *phn* operon demonstrated that *phn*O was not required for phosphonate utilization, further supporting the proposed regulatory function for this gene (Metcalf et al., J. Bacteriol. 173:587-600, 1991), at least for the phosphonates tested. Homologous *phn* sequences have been identified from other bacteria, including a gene substantially similar to *E. coli phn*O, isolated from *S. griseus*, using nucleotide sequences deduced from those in the *E. coli phn*O gene (Jiang et al., J. Bacteriol. 177:6411-6421, (1995); McGrath et al., Eur. J. Biochem. 234:225-230, (1995); Ohtaki et al., Actinomyceteol. 8:66-68, (1994)). However, no function other than as a regulatory factor has been proposed for *phn*O. A regulatory role for *phn*O in the CP lyase operon has been cited again in a recent review (Berlyn, Microbiol. Molec. Biol. Rev. 62:814-984, 1998).

Advances in molecular biology, and in particular in plant sciences in combination with recombinant DNA technology, have enabled the construction of recombinant plants which contain nonnative genes of agronomic importance. Furthermore, when incorporated into and expressed in a plant, such genes desirably confer some beneficial trait or characteristic to the recombinant plant. One such trait is herbicide resistance. A recombinant plant capable of growth in the presence of a herbicide has a tremendous advantage over herbicide-susceptible species. In addition, herbicide tolerant plants provide a more cost effective means for agronomic production by educing the need f - tillage to control weeds and volunteers.

Chemical herbicides have been used for decades to inhibit plant metabolism, particularly for agronomic purposes as a means for controlling weeds or volunteer plants in fields of crop

plants. A class of herbicides which have proven to be particularly effective for these purposes are known as phosphonates or phosphonic acid herbicides. Perhaps the most agronomically successful phosphonate herbicide is glyphosate (N-phosphono-methyl-glycine).

Recombinant plants have been constructed which are tolerant to the phosphonate herbicide glyphosate. When applied to plants, glyphosate is absorbed into the plant tissues and inhibits aromatic amino acid formation, mediated by an inhibition of the activity of the plastid-localized 5-enolpyruvyl-3-phosphoshikimic acid synthase enzyme, also known as EPSP synthase or EPSPS, an enzyme generally thought to be unique to plants, bacteria and fungi. Recombinant plants have been transformed with a bacterial EPSPS enzyme which is much less sensitive to glyphosate inhibition. Therefore, plants expressing this bacterial EPSPS are less sensitive to glyphosate, and are often characterized as being glyphosate tolerant. Therefore, greater amounts of glyphosate can be applied to such recombinant plants, ensuring the demise of plants which are susceptible or sensitive to the herbicide. However, other genes have been identified which, when transformed into a plant genome, encoding enzymes which also provide glyphosate tolerance. One such enzyme has been described as GOX, or glyphosate-oxidoreductase. GOX functions in providing protection to plants from the phosphonate herbicide glyphosate by catalyzing the degradation of glyphosate to aminomethyl phosphonic acid (AMPA) and glyoxylate. AMPA produced as a result of glyphosate degradation can cause bleaching and stunted or depressed plant growth, among other undesireable characteristics. Many plant species are also sensitive to exogeneously applied AMPA, as well as to endogenous AMPA produced as a result of GOX mediated glyphosate herbicide degradation. No method has been described which discloses the protection of plants from applications of phosphonate herbicides such as AMPA.

Barry et al. (US Patent No. 5,633,435) disclose genes encoding EPSP synthase enzymes which are useful in producing transformed bacteria and plants which are tolerant to glyphosate as a herbicide, as well as the use of such genes as a method for selectively controlling weeds in a planted transgenic crop field. Barry et al. (US Patent No. 5,463,175) disclose genes encoding glyphosate oxidoreductase (GOX) enzymes useful in producing transformed bacteria and plants which degrade glyphosate herbicide as well as crop plants which are tolerant to glyphosate as a herbicide. Barry et al. (US Pat No. 5,463,175) disclosed the formation of AMPA as a product of GOX mediated glyphosate metabolism.AMPA has been reported to be much less phytotoxic than glyphosate for most plant species (Franz, 1985) but not for all plant species (Maier, 1983;

Tanaka et al., 1986). Co-expression of a gene encoding a protein capable of neutralizing or metabolizing AMPA produced by glyphosate degradation would provide a substantial improvement over the use of GOX alone. Thus, a method for overcoming sensitivity to AMPA formation as a result of glyphosate degradation, or a method for resistance to AMPA when used as a herbicide or as a selective agent in plant transformation methods, would be useful for providing enhanced or improved herbicide tolerance in transgenic plants and in other organisms sensitive to such compounds.

The use of glyphosate as a chemical gametocide has been described (U.S. Patent No. 4,735,649). Therein, it is disclosed that glyphosate can, under optimal conditions, kill about 95% of male gametes, while leaving about 40-60% of the female gametes capable of fertilization. In addition, a stunting effect was typically observed at the application levels disclosed, shown by a reduction in the size of the plant and by a minor amount of chlorosis. Thus, a major drawback of using glyphosate as a gametocide, as is generally true with most gametocides, is the phytotoxic side effects resulting from lack of sufficient selectivity for male gametes. These phytotoxic manifestations may be effectuated by AMPA production in transgenic plants expressing GOX after treatment with glyphosate. Therefore, it would be advantageous to provide a method for preventing the stunting effect and chlorosis as side effects of using glyphosate as a gametocide in transgenic plants expressing GOX. Furthermore, a more effective method would optimally kill more than 95% of male gametes or prevent male gametes from maturing and would leave greater than 60% of female gametes substantially unaffected. It is believed that tissue specific co-expression of GOX with a transacylase gene encoding an enzyme capable of N-acylation of AMPA would achieve this goal.

It has now been discovered that the *E. coli phn*O gene encodes an enzyme having transacylase, acyltransferase, or Acyl-CoA transacylase activity in which a preferred substrate is a phosphonate displaying a terminal amine, and in particular amino-methyl-phosphonic acid (AMPA). The transfer of an acyl group from an Acyl-CoA to the free terminal amine of AMPA results in the formation of an N-acylated AMPA. Plants are not known to acylate AMPA to any great extent, and some plants have been shown to be sensitive to AMPA and insensitive to acyl-AMPA. Thus, expression of *phn*O in plants would be useful in enhancing the phosphonate herbicide tolerance, particularly when AMPA is used as a herbicide or selective agent in plant

transformation, and more particularly when glyphosate is used as a herbicide in combination with recombinant plants expressing a GOX gene.

Summary of the Invention

Briefly therefore the present invention is directed to a composition of matter comprising a novel class of genes which encode proteins capable of N-acylation of phosphonate compounds and to methods of using these genes and encoded proteins for improving plant tolerance to phosphonate herbicides. The present invention is also directed to a method for selecting recombinant plants and microbes transformed with genes encoding proteins which are capable of N-acylation of phosphonate compounds, and to peptides which are capable of N-acylation of the compound N-amino-methyl-phosphonic acid (N-AMPA) and other related phosphonate compounds. In addition, the present invention is also directed to a method for using plants transformed with transacylase genes to prevent self-fertilization or to a method for enhancing hetero-fertilization in plants.

Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of producing stably transformed herbicide tolerant recombinant plants which have inserted into their genomes a polynucleotide sequence encoding a desired gene product, preferably an N-acyl-transferase enzyme. The polynucleotide sequence preferably is composed of a cassette containing a promoter sequence which is functional in plants and which is operably linked 5' to a structural DNA sequence which, when transcribed into an RNA sequence, encodes an N-acyl-transferase enzyme peptide. The promoter sequence can be heterologous with respect to the structural DNA sequence and causes sufficient expression of the transferase enzyme in plant tissue to provide herbicide tolerance to the plant transformed with the polynucleotide sequence. The structural sequence is preferably operably linked 3' to a 3' non-translated polyadenylation sequence which functions in plants, and which when transcribed into RNA along with the structural sequence causes the addition of a polyadenylated nucleotide sequence to the 3' end of the transcribed RNA. Expression of the structural DNA sequence produces sufficient levels of the acyltransferase enzyme in the plant tissue to enhance the herbicide tolerance of the transformed plant.

As a further embodiment, the structural DNA sequence may also contain an additional 5' sequence encoding an amino-terminal peptide sequence which functions in plants to target the

peptide produced from translation of the structural sequence to an intracellular organelle. This additional coding sequence is preferably linked in-frame to the structural sequence encoding the acyltransferase enzyme. The amino terminal peptide sequence can be either a signal peptide or a transit peptide. The intracellular organelle can be a chloroplast, a mitochondrion, a vacuole, endoplasmic reticulum, or other such structure. The structural DNA sequence may also be linked to 5' sequences such as untranslated leader sequences (UTL's), intron sequences, or combinations of these sequences and the like which may serve to enhance expression of the desired gene product. Intron sequences may also be introduced within the structural DNA sequence encoding the acyltransferase enzyme. Alternatively, chloroplast or plastid transformation can result in localization of an acyltransferase coding sequence and enzyme to the chloroplast or plastid, obviating the requirement for nuclear genome transformation, expression from the nuclear genome, and subsequent targeting of the gene product to a subcellular organelle.

Preferably, the recombinant plant expresses a gene encoding an enzyme which catalyzes the formation of AMPA. AMPA formation can result from the metabolism of a naturally occurring precursor, from a precursor such as glyphosate provided to the plant, or can result from the formation of AMPA through some catabolic pathway. Co-expression of GOX along with AMPA acyltransferase expression provides a plant which is surprisingly more resistant to certain phosphonate herbicides. However, one embodiment allowing plants transformed with only an N-acyltransferase to grow in the presence of AMPA or similar or related compounds would provide a useful selective method for identifying genetically transformed plants, callus, or embryogenic tissues.

In accordance with another aspect of the present invention is the provision of a method for selectively enhancing or improving herbicide tolerance in a recombinant plant which has inserted into its nuclear, chloroplast, plastid or mitochondrial genome a cassette comprised of a polynucleotide sequence which encodes an N-acyl-transferase enzyme.

A further embodiment encompasses the improvement of a method for selectively enhancing herbicide tolerance in a transformed plant expressing a GOX gene which encodes a glyphosate oxidoreductase enzyme expressed in the same plants in which an acyltransferase enzyme is produced.

In accordance with another aspect of the present invention is the provision of a method for producing a genetically transformed herbicide tolerant plant by inserting into a genome of a plant cell a cassette comprising a polynucleotide sequence which encodes an N-acyl-transferase enzyme.

A further embodiment encompasses the improvement of a method for producing a genetically transformed herbicide tolerant plant from a plant cell expressing a GOX gene which encodes a glyphosate oxidoreductase enzyme expressed in the same plant cell in which an acyltransferase enzyme is produced.

In any of the foregoing embodiments, the herbicide tolerant plant or plant cell can be selected from the group consisting of corn, wheat, cotton, rice, soybean, sugarbeet, canola, flax, barley, oilseed rape, sunflower, potato, tobacco, tomato, alfalfa, lettuce, apple, poplar, pine, eucalyptus, acacia, poplar, sweetgum, radiata pine, loblolly pine, spruce, teak, alfalfa, clovers and other forage crops, turf grasses, oilpalm, sugarcane, banana, coffee, tea, cacao, apples, walnuts, almonds, grapes, peanuts, pulses, petunia, marigolds, vinca, begonias, geraniums, pansy, impatiens, oats, sorghum, and millet.

In accordance with another aspect of the present invention is the provision of a peptide capable of N-acylation of the compound N-aminomethylphosphonic acid (N-AMPA or AMPA) or other such compounds which are capable of causing phytotoxic effects when applied to, introduced into, or produced by plant metabolisms. One such peptide is N-aminomethylphosphonic acid transacylase (AAT) derived from expression of an *E. coli phn*O structural gene sequence. Other peptides similar in structure and function to the *E. coli phn*O gene product are also contemplated.

Another aspect of the present invention is the provision of a method for selecting cells transformed with a vector containing an acyltransferase gene expressing an enzyme capable of N-acylation of AMPA and like compounds. The method includes the steps of transforming a population of cells with the vector, and isolating and purifying the transformed cells from non-transformed cells in the population after selecting for the transformed cells by incubation in the presence of amounts of AMPA sufficient to be inhibitory to the growth or viability of any non-transformed cells. The transformed cells can be bacterial, plant or fungal cells. Bacterial cells can be members of any of the families encompassed by *Enterobacteraceae*, *Mycobacteraceae*, *Agrobacteraceae*, and *Actinobacteraceae*, among others. Fungal cells can be members of

Ascomycota, *Basidiomycota*, etc. Plant cells can be derived from any member of the *Plantae* family.

A further embodiment of the present invention provides for a method for producing a plant from a tissue, a cell, or other part of a plant which was derived from a plant transformed with an acyltransferase gene, a *phn*O gene, a *gox* gene, a gene in which GOX and acyltransferase peptides are produced from a translational fusion or a transcriptional fusion, or a polycistronic gene which encodes GOX and acyltransferase peptides.

A further embodiment of the present invention provides for a method for producing plants which express all or a portion of a *phnO* gene or similar acyltransferase gene, or a GOX gene as an antisense gene in a tissue specific manner.

Other aspects also include reagents such as antibodies directed to AMPA acyltransferase, and polynucleotides for use in identifying acyltransferase gene sequences. These reagents can be included in kits containing AMPA acyltransferase, polynucleotides which are or are complimentary to an AMPA acyltransferase gene sequence, polynucleotides for use in thermal amplification of an AMPA acyltransferase gene sequence, antibodies directed to AMPA acyltransferase for the detection of AMPA acyltransferase in the laboratory or in the field, and any other reagents necessary for use in kit form as well as for use in other assays contemplated herein.

A further object of the present invention is to provide a method for using phosphonate herbicides as chemical hybridizing agents. The method allows for selective gametocidal effects and for the production of male sterile plants. Such plants may be engineered so that *gox* or *phn*O, or *gox* and *phn*O fail to be expressed in plant tissues required for reproduction, causing sensitivity to applied phytotoxic compounds which inhibit formation of mature gamete structures.

Brief Description of the Drawings

Figure 1 illustrates a [^{14}C] isotope detection HPLC chromatogram representing a sample of a dosing solution containing only [^{14}C] glyphosate (11.3 minutes, 98.8%), and trace amounts of [^{14}C] AMPA (5.8 minutes, 0.16%) and an unidentified [^{14}C] material (10.2 minutes, 1%).

Figure 2 illustrates an HPLC profile of a mixture of standards of the observed radioactive metabolites [^{14}C] AMPA, [^{14}C] glyphosate, and N-acetyl-[^{14}C]-AMPA, as well as the impurity identified as N-acetyl-N-methyl-[^{14}C]-AMPA.

Figure 3 illustrates a representative HPLC profile of an extract from a corn callus tissue transformed with GOX and AMPA acetyltransferase, and treated with [^{14}C] glyphosate. The peaks indicate [^{14}C] glyphosate (10.8 minutes, 92.5% of total observed [^{14}C]), [^{14}C] AMPA primarily generated by GOX mediated glyphosate degradation (5.98 minutes, 1.71% of total observed [^{14}C]), and N-acetyl-[^{14}C]AMPA produced from acylation of [^{14}C] AMPA mediated by recombinant AMPA acyltransferase expressed within callus tissue (13.29 minutes, 4.54% total observed [^{14}C]).

Figure 4 illustrates plasmid pMON17261.

Figure 5 illustrates plasmid pMON32571.

Figure 6 illustrates plasmid pMON32936.

Figure 7 illustrates plasmid pMON32946.

Figure 8 illustrates plasmid pMON32948.

Detailed Description of the Invention

The following detailed description of the invention is provided to aid those skilled in the art in practicing the present invention. Even so, the following detailed description should not be construed to unduly limit the present invention as modifications and variations in the embodiments discussed herein may be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

Many words and phrases are well known in the art of molecular biology, microbiology, protein chemistry, and plant sciences and generally have their plain and ordinarily understood meaning, otherwise to be taken in context. However, the following words and phrases as used herein have the meanings generally set forth below.

AMPA acyltransferase. As used herein, AMPA acyltransferase refers to an enzyme which functions in transferring an acyl chemical group from an acylcarrier compound such as coenzyme A, which is well known and abbreviated in the biological and chemical arts as CoA. In particular, an AMPA acyltransferase transfers an acyl chemical group from an acylcarrier to the free amino group of aminomethylphosphonate, well known to be a byproduct of glyphosate

oxidoreductase mediated glyphosate metabolism. AMPA acyltransferase (AAT), which herein may also be known as AMPA acetyltransferase, AMPA transacylase, or acetyl-AMPA synthase (AAS), has been shown herein to be capable of acetyl transferase activity, propionyl transferase activity, malonyl transferase activity, and succinyl transferase activity. Thus, any biologically functional equivalent of these compounds (acetyl, propionyl, malonyl, or succinyl) which serves as an acyl-carrier-form of substrate capable of functioning with an AMPA acyltransferase enzyme is within the scope of the present invention. One AMPA acyltranferase which has been identified, and shown by example herein to function according to the description contained herein, has previously been referred to in the art as PhnO, a protein encoded by the *phn*O gene within the *E. coli phn* operon.

Biological functional equivalents. As used herein such equivalents with respect to the AMPA-acyltransferase proteins of the present invention are peptides, polypeptides and proteins that contain a sequence or moiety exhibiting sequence similarity to the novel peptides of the present invention, such as PhnO, and which exhibit the same or similar functional properties as that of the polypeptides disclosed herein, including transacylase activity. Biological equivalents also include peptides, polypeptides and proteins that react with, *i.e.* specifically bind to antibodies raised against PhnO and that exhibit the same or similar transacylase activity, including both monoclonal and polyclonal antibodies.

Biological functional equivalents as used herein with respect to genes encoding acyltransferases are polynucleotides which react with the polynucleotide sequences contemplated and described herein, i.e. which are capable of hybridizing to a polynucleotide sequence which is or is complementary to a polynucleotide encoding an acyltransferase which functions in transacylation of AMPA or which encode substantially similar acyltransferase proteins contemplated and described herein. A protein which is substantially similar to the proteins described herein is a biological functional equivalent and exhibits the same or similar functional properties as that of the polypeptides disclosed herein, including improved herbicide tolerance or improved herbicide resistance. Biological equivalent peptides contain a sequence or moiety such as one or more active sites which exhibit sequence similarity to the novel peptides of the present invention, such as PhnO. Biological equivalents also include peptides, polypeptides, and proteins that react with, i.e. which specifically bind to antibodies raised against PhnO and PhnO-

like peptide sequences and which exhibit the same or similar improvement in herbicidal tolerance or resistance, including both monoclonal and polyclonal antibodies.

Chloroplast or plastid localized, as used herein, refers to a biological molecule, either polynucleotide or polypeptide, which is positioned within the chloroplast or plastid such that the molecule is isolated from the cellular cytoplasmic milieu, and functions within the chloroplast or plastid cytoplasm to provide the effects claimed in the instant invention. Localization of a biological molecule to the chloroplast or plastid can occur, with reference to polynucleotides, by artificial mechanical means such as electroporation, mechanical microinjection, or by polynucleotide coated microprojectile bombardment, or with reference to polypeptides, by secretory or import means wherein a natural, non-naturally occurring, or heterologous plastid or chloroplast targeting peptide sequence is used which functions to target, insert, assist, or localize a linked polypeptide into a chloroplast or plastid.

Event refers to a transgenic plant or plant tissue derived from the insertion of foreign DNA into one or more unique sites in the nuclear, mitochondrial, plastid or chloroplast DNA.

Expression: The combination of intracellular processes, including transcription, translation, and other intracellular protein and RNA processing and stabilization functions, which a coding DNA molecule such as a structural gene is subjected to in order to produce a gene product.

Non-naturally occurring gene: A non-naturally occurring acyl-transferase gene of the present invention contains genetic information encoding a plant functional RNA sequence, but preferably is a gene encoding an acyl-transferase protein, whether naturally occurring or a variant of a naturally occurring protein, prepared in a manner involving any sort of genetic isolation or manipulation. This includes isolation of the gene from its naturally occurring state, manipulation of the gene as by codon modification, site specific mutagenesis, truncation, introduction or removal of restriction endonuclease cleavage sites, synthesis or resynthesis of a naturally occurring sequence encoding an acyltransferase of the present invention by *in vitro* methodologies such as phosphoramidite chemical synthesis methods, etc., thermal amplification methods such as polymerase chain reaction, ligase chain reaction, inverted polymerase reaction, and the like etc., and any other manipulative or isolative method.

Operably Linked: Nucleic acid segments connected in frame so that the properties of one influence the expression of the other. For example, a promoter sequence having properties

of polymerase loading, binding, and initiation of transcription functions influences the expression of sequences which are linked to the promoter.

Plant-Expressible Coding Regions: Coding regions which are expressible, i.e can be transcribed and/or translated *in planta*, because they contain typical plant regulatory elements to facilitate the expression of a gene of interest.

Plastid Transit Peptide: Any amino acid sequence useful in targeting or localizing a linked amino acid, such as a protein fusion, to a subcellular compartment or organelle such as a plastid or chloroplast. Amino acid sequences which facilitate entry into a mitochondria are not altogether unlike or dissimilar from plastid transit peptides, and are also described as transit peptides, but fail to function for targeting peptide sequences to plastid or chloroplast organelles.

Progeny of a transgenic plant includes any offspring or descendant of the transgenic plant which contains at least one heterologous or trans-gene, or any subsequent plant derived from the transgenic plant which has the transgene in its lineage. Progeny is not limited to one generation, but rather encompasses the descendants of the transgenic plant so long as they contain or express the desired transgene. Seeds containing transgenic embryos as well as seeds from the transgenic plants and their offspring or descendants are also important parts of the invention. Transgenic cells, tissues, seeds or plants which contain a desired transgene are progeny of the original transgenic cells, tissue, or plant.

Promoter: A recognition site on a DNA sequence or group of DNA sequences that provides an expression control element for a structural gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

R_0 is the primary regenerant plant derived from transformation of plant tissue or cells in culture. Subsequent progeny or generations derived from the R_0 are referred to as R_1 (first generation), R_2 (second generation), *etc.*

Regeneration: The process of producing a whole plant by growing a plant from a plant cell or plant tissue (*e.g.*, plant protoplast or explant).

Structural Coding Sequence refers to a DNA sequence that encodes a peptide, polypeptide, or protein that is produced following transcription of the structural coding sequence to messenger RNA (mRNA), followed by translation of the mRNA to produce the desired peptide, polypeptide, or protein product.

Structural gene: A gene that is expressed to produce a polypeptide.

Substantial homology: As this term is used herein, substantial homology refers to nucleic acid sequences which are from about 40 to about 65 percent homologous, from about 66 percent homologous to about 75 percent homologous, from about 76 percent homologous to about 86 percent homologous, from about 87 percent homologous to about 90 percent homologous, from about 91 percent homologous to about 95 percent homologous, and from about 96 percent homologous to about 99 percent homologous to a reference polynucleotide sequence, such as either an *E. coli phn*O gene sequence. A first polynucleotide molecule which is substantially homologous to a second polynucleotide molecule is or is complimentary to the second polynucleotide such that the first polynucleotide molecule hybridizes to the second polynucleotide molecule or its complementary sequence under stringent hybridization conditions, with stringency being defined as the optimum concentration of salt and temperature required to bring about hybridization of a first polynucleotide to a second polynucleotide. Methods for varying stringency are well known in the art but may be referenced in Sambrook et al., Eds., Molecular Cloning: A Laboratory Manual, Second Edition, 1989, Cold Spring Harbor Press; or Ausubel et al, Eds., Short Protocols in Molecular Biology, Third Edition, 1995, John Wiley and Sons, Inc. Polypeptides which are believed to be within the scope if the present invention are those which are from about 40 to about 65 percent similar, from about 66 percent similar to about 75 percent similar, from about 76 percent similar to about 86 percent similar, from about 87 percent similar to about 90 percent similar, from about 91 percent similar to about 95 percent similar, and from about 96 percent similar to about 99 percent similar to a reference polypeptide sequence, preferably to an *E. coli* PhnO peptide sequence.

Terminator: As used herein with respect to plant specific sequences intended for *in planta* expression, the 3′ end transcription termination and polyadenylation sequence.

Transformation is a process of introducing an exogenous polynucleotide sequence, such as a plasmid or viral vector or a recombinant polynucleotide molecule, into a cell, protoplast, plastid or chloroplast, or mitochondria in which the exogenous polynucleotide sequence is either incorporated into an endogenous polynucleotide sequence contained within the cell, or is capable of autonomous replication. A transformed cell is a cell which has been altered by the introduction of one or more exogenous polynucleotide molecules into that cell. A stably transformed cell is a transformed cell which has incorporated all or a portion of the exogenous polynucleotide into the cells' nuclear, mitochondrial, or plastid or chloroplast genomic material

such that the exogenous polynucleotide confers some genotypic or phenotypic trait or traits to that cell and to the progeny of the transformed cell, measured by the detection of the exogenously introduced polynucleotide, the mRNA or protein product of the exogenous polynucleotide, a metabolite not normally produced by or found within the cell in the absence of the exogenous polynucleotide, or a visual inspection of the cell, plant tissue, or plants derived from the transformed cell.

Transgene: A transgene is a polynucleotide sequence which has been transferred to a cell and comprises an expression cassette containing a structural gene sequence encoding a desired polypeptide. The transgene is capable of being expressed when in a recipient transformed cell, tissue, or organism. This may include an entire plasmid or other vector, or may simply include the plant functional coding sequence of the transferred polynucleotide. A transgenic cell is any cell derived from or regenerated from a transformed cell, including the initially transformed cell. Exemplary transgenic cells include plant callus tissue derived from a transformed plant cell and particular cells such as leaf, root, stem, meristem, and other somatic tissue cells, or reproductive or germ line and tapetal cells obtained from a stably transformed transgenic plant. A transgenic event is a plant or progeny thereof derived from the insertion of at least one exogenous polynucleotide into the nuclear, plastidic, or mitochondrial genome of a plant cell or protoplast. A transgenic plant is a plant or a progeny thereof which has been genetically modified to contain and express heterologous polynucleotide sequences as proteins or as RNA or DNA molecules not previously a part of the plant composition. As specifically exemplified herein, a transgenic cotton plant, for example, is genetically modified to contain and express at least one heterologous DNA sequence operably linked to and under the regulatory control of transcriptional and translational control sequences which function in plant cells or tissue or in whole plants. A transgenic plant may also be referred to as a transformed plant. A transgenic plant also refers to progeny of the initial transgenic plant where those progeny contain and are capable of expressing the heterologous coding sequence under the regulatory control of the plant expressible transcriptional and translational control sequences described herein. A transgenic plant can produce transgenic flowers, seeds, bulbs, roots, tubers, fruit, and pollen and the like and can be crossed by conventional breeding means with compatible lines of plants to produce hybrid transgenic plants.

Vector: A DNA or other polynucleotide molecule capable of replication in a host cell and/or to which another DNA or other polynucleotide sequence can be operatively linked so as to bring about replication of the linked sequence. A plasmid is an exemplary vector.

In accordance with the present invention, it has been discovered that plants can produce a phytotoxic compound when transformed with certain genes encoding enzymes capable of degrading glyphosate. In particular, glyphosate oxidoreductase (GOX) mediated metabolism of glyphosate produces a phytotoxic compound identified as N-aminomethyl-phosphonate (AMPA). Other studies have shown that an N-acylated derivative of AMPA, N-acyl-aminomethyl-phosphonate (N-acyl-AMPA or acyl-AMPA), is much less phytotoxic to most plant species. Enzymes have been identified which are able to covalently modify AMPA through an acylation mechanism, resulting in the formation of N-acyl-AMPA. One enzyme in particular causes exogeneously applied AMPA to be N-acetylated. In plants expressing this enzyme along with GOX, phytotoxic AMPA effects are not observed.

The inventions contemplated herein take advantage of recombinant polynucleotide cassettes comprised of elements for regulating gene expression into which sequences, such as structural genes encoding useful proteins, can be inserted. Insertion of such sequences into an expression cassette is preferably accomplished using restriction endonucleases well known in the art, however other methods for insertion are known. For example, site specific recombination methods are effective for inserting desired sequences into such expression cassettes. Expression cassettes contain at least a plant operable promoter for use in initiating the production of a messenger RNA molecule from which the useful protein is translated. Cassettes also contain plant operable sequences, identified as 3' sequences, which function in terminating transcription and provide untranslated sequences which are 3' polyadenylated. Thus, an expression cassette intended for use in plants should contain at least a promoter sequence linked at its 3' end to a 3' transcription termination and polyadenylation sequence. Preferably, a polycloning sequence or linker sequence containing one or more unique restriction endonuclease cleavage sites is present bridging the promoter and 3' sequence for convenient insertion of structural gene sequences and other elements. An expression cassette intended for use in plants also preferably contains a 5' untranslated sequence inserted between the promoter and the 3' sequence. 5' untranslated sequences (UTL's) have been shown to enhance gene expression in plants. Introns are also contemplated as sequences which may be present in such expression cassettes of the present

invention. The presence of plant operable introns has also been shown, in maize in particular, to enhance gene expression in certain plant species. Introns may be present in an expression cassette in any number of positions along the sequence of the cassette. This can include positions between the promoter and the 3' termination sequence and/or within a structural gene. There may be more than one intron present in an expression cassette, however for the purposes of the contemplated inventions herein, it is preferred that introns be present when expression cassettes are used in monocotyledonous plants and plant tissues. Enhancer sequences are also well known in the art and may be present, although not necessarily as a part of an expression cassette, as enhancer sequences are known to function when present upstream or downstream or even at great distances from a promoter driving expression of a gene of interest.

The expression of a gene localized to the plant nuclear genome and which exists in double-stranded DNA form involves transcription to produce a primary messenger RNA transcript (mRNA) from one strand of the DNA by RNA polymerase enzyme, and the subsequent processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' non-translated polynucleotide sequence which adds polyadenylate nucleotides to the 3' end of the RNA. Transcription of DNA into mRNA is regulated by a sequence of DNA usually referred to as the "promoter". The promoter comprises a sequence of bases that signals RNA polymerase to associate with the DNA and to initiate the transcription of mRNA using the template DNA strand to make a corresponding complementary strand of RNA.

Those skilled in the art will recognize that there are a number of promoters which are active in plant cells, and have been described in the literature. Such promoters may be obtained from plants, plant viruses, or plant commensal, saprophytic, symbiotic, or pathogenic microbes and include, but are not limited to, the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*), the cauliflower mosaic virus (CaMV) 19S and 35S promoters, the light-inducible promoter from the small subunit of ribulose 1,5-bisphosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide), the rice *Act1* promoter, the Figwort Mosaic Virus (FMV) 35S promoter, the sugar cane bacilliform DNA virus promoter, the ubiquitin promoter, the peanut chlorotic streak virus promoter, the comalina yellow virus promoter, the chlorophyll a/b binding protein promoter, and meristem enhanced promoters Act2, Act8, Act11 and EF1a and the like. All of these promoters have been used to create various types of DNA constructs which have been expressed in plants

(see e.g., McElroy et al., 1990; Barry and Kishore, USP 5,463,175) and which are within the scope of the present invention. Chloroplast and plastid specific promoters, chloroplast or plastid functional promoters, and chloroplast or plastid operable promoters are also envisioned. It is preferred that the particular promoter selected should be capable of causing sufficient in-planta expression to result in the production of an effective amount of acyltransferase to render a plant substantially tolerant to phosphonate herbicides and products of phosphonate herbicide metabolism. The amount of acyltransferase required to provide the desired tolerance may vary with the plant species.

One set of preferred promoters are constitutive promoters such as the CaMV35S or FMV35S promoters that yield high levels of expression in most plant organs. Enhanced or duplicated versions of the CaMV35S and FMV35S promoters are particularly useful in the practice of this invention (Kay et al, 1987; Rogers, USP 5,378, 619). In addition, it may also be preferred to bring about expression of the acyltransferase gene in specific tissues of the plant, such as leaf, stem, root, tuber, seed, fruit, etc., and the promoter chosen should have the desired tissue and developmental specificity. Therefore, promoter function should be optimized by selecting a promoter with the desired tissue expression capabilities and approximate promoter strength and selecting a transformant which produces the desired herbicide tolerance in the target tissues. This selection approach from the pool of transformants is routinely employed in expression of heterologous structural genes in plants since there is variation between transformants containing the same heterologous gene due to the site of gene insertion within the plant genome. (Commonly referred to as "position effect"). In addition to promoters which are known to cause transcription (constitutive or tissue-specific) of DNA in plant cells, other promoters may be identified for use in the current invention by screening a plant cDNA library for genes which are selectively or preferably expressed in the target tissues and then determine the promoter regions.

It is preferred that the promoters utilized have relatively high expression in all meristematic tissues in addition to other tissues inasmuch as it is now known that phosphonate herbicides can be translocated and accumulated in this type of plant tissue. Alternatively, a combination of chimeric genes can be used to cumulatively result in the necessary overall expression level of acyltransferase enzyme to result in the herbicide tolerant phenotype. A promoter which provides relatively high levels of expression can cause the production of a

desired protein to *in planta* levels ranging from 0.1 milligrams per fresh weight gram of plant tissue, to 0.5 milligrams per fresh weight gram of plant tissue, to 1.0 milligrams per fresh weight gram of plant tissue, to 2.0 or more milligrams per fresh weight gram of plant tissue. The *in planta* levels of a desired protein in genetically isogenic crops in a field can range across a spectrum, but generally the levels fall within 70% of a mean, more preferably within 50% of a mean, and even more preferably within 25% of a mean for all plants analyzed in a given sample.

The promoters used in the DNA constructs (i.e. chimeric plant genes) of the present invention may be modified, if desired, to affect their control characteristics. For example, the CaMV35S promoter may be ligated to the portion of the *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase small subunit gene (ssRUBISCO) that represses the expression of ssRUBISCO in the absence of light, to create a promoter which is active in leaves but not in roots. The resulting chimeric promoter may be used as described herein. For purposes of this description, the phrase "CaMV35S" promoter thus includes variations of CaMV35S promoter, e.g., promoters derived by means of ligation with operator regions, random or controlled mutagenesis, et cetera. Furthermore, the promoters may be altered to contain multiple "enhancer sequences" to assist in elevating gene expression. Examples of such enhancer sequences have been reported by Kay et al. (1987).

One RNA produced by a DNA construct of the present invention also contains a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. The nontranslated or 5' untranslated leader sequence (NTR or UTR) can be derived from an unrelated promoter or coding sequence. For example, the 5' non-translated regions can also be obtained from viral RNA's, from suitable eucaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs, as presented in one of the following examples, wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. Examples of plant gene leader sequences which are useful in the present invention are the wheat chlorophyll a/b binding protein (cab) leader and the petunia heat shock protein 70 (hsp70) leader (Winter et al., 1988).

For optimal expression in monocotyledonous plants, an intron should also be included in the DNA expression construct. This intron would typically be placed near the 5' end of the mRNA in untranslated sequence. This intron could be obtained from, but not limited to, a set of

introns consisting of the maize hsp70 intron (Brown et al., US Patent No. 5,424,412; 1995) or the rice *Act1* intron (McElroy et al., 1990).

When more than one expression cassette in included within a plasmid or other polynucleotide construct, a first expression cassette comprising a DNA molecule typically contains a constitutive promoter, a structural DNA sequence encoding a glyphosate oxidoreductase enzyme (GOX), and a 3' non-translated region. A second expression cassette comprising a DNA molecule typically contains a constitutive promoter, a structural DNA sequence encoding an N-acyl-transferase enzyme which is capable of reacting with AMPA to produce N-acyl-AMPA, and a 3' non-translated region. Additional expression cassettes comprising a DNA molecule are also envisioned. For example, genes encoding insecticidal or fungicidal activities, drought or heat tolerance, antibiotic compounds, pharmaceutical compounds or reagents such as tumor suppressor proteins or antibody components, biopolymers, other commercially useful compounds and the like may also be expressed in the plants envisioned by the present invention, along with genes which provide increased herbicide tolerance. A number of constitutive promoters which are active in plant cells have been described. Suitable promoters for constitutive expression of either GOX or an N-acyl-transferase include, but are not limited to, the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al. 1985), the Figwort mosaic virus (FMV) 35S (Sanger et al. 1990), the sugarcane bacilliform DNA virus promoter (Bouhida et al., 1993), the commelina yellow mottle virus promoter (Medberry and Olszewski 1993), the light-inducible promoter from the small subunit of the ribulose-1,5-bis-phosphate carboxylase (ssRUBISCO) (Coruzzi et al., 1984), the rice cytosolic triosephosphate isomerase (TPI) promoter (Xu et al. 1994), the adenine phosphoribosyltransferase (APRT) promoter of *Arabidopsis* (Moffatt et al. 1994), the rice actin 1 gene promoter (Zhong et al. 1996), and the mannopine synthase and octopine synthase promoters (Ni et al. 1995). All of these promoters have been used to create various types of plant-expressible recombinant DNA constructs. Comparative analysis of constitutive promoters by the expression of reporter genes such as the *uidA* (β-glucuronidase) gene from *E. coli* has been performed with many of these and other promoters (Li et al. 1997; Wen et al. 1993).

Promoters used in the second cassette comprising a DNA molecule can be selected to control or limit specific expression where cell lethality is desired. In a preferred embodiment, the promoter will be capable of directing expression exclusively or primarily in tissues critical

for plant survival or plant viability, while limiting expression of the second cassette comprising a DNA molecule in other nonessential tissues. For example, tissues which differentiate into pollen development or terminal tissues such as the pollen itself, the tapetal cell layer of the anther, or the anther tissues. Alternatively, plant promoters capable of regulating the expression of genes in particular cell and tissue types are well known. Those that are most preferred in the embodiments of this invention are promoters which express specifically during the development of the male reproductive tissue or in pollen at levels sufficient to produce inhibitory RNA molecules complementary to the sense RNA transcribed by the constitutive promoter of the first expression cassette comprising a DNA molecule. Examples of these types of promoters include the TA29 tobacco tapetum-specific promoter (Mariani et al. 1990), the PA1 and PA2 chalcone flavonone isomerase promoters from petunia (van Tunen et al. 1990), the SLG gene promoter from *Brassica oleracea* (Heizmann et al. 1991), and LAT gene promoters from tomato (Twell et al. 1991).

Anther and pollen-specific promoters from rice have been isolated. Examples include the Osg6B promoter, which was shown to drive expression of the β-glucuronidase gene in transgenic rice in immature anthers. No activity was detected in other tissues of spikelets, leaves or roots (Yokoi et al. 1997). The PS1 pollen-specific promoter from rice has been shown to specifically express the β-glucuronidase gene in rice pollen (Zou et al., 1994). Additional rice genes have been identified that specifically express in the anther tapetum of rice (Tsuchiya et al. 1994, Tsuchiya et al. 1997). The isolation of additional genes expressed predominantly during anther development in rice can be performed, for example, by construction of a cDNA library to identify anther specific clones (Qu et al.).

Those skilled in the art are aware of the approaches used in the isolation of promoters which function in plants, and from genes or members of gene families that are highly expressed in particular plant tissues such as in roots, shoots, meristem, leaves, flowers, fruits, in pollen, or in plant cell types involved in the production of pollen (Stinson et al. 1987; Brown and Crouch. 1990; McCormick et al. 1989). Further examples of tissue specific promoters include the promoter for the exopolygalacturonase gene of maize (Dubald, et al. 1993) and the promoter for the Zm-c13 mRNA (Hanson, et al. 1989). Promoters which have been shown to preferentially express in tomato pollen are the LAT52 and LAT59 promoters (Twell et al. 1991). A portion of the maize pZtap promoter sequence (psgB6-1) was disclosed in U.S. Patent 5,470,359.

A recombinant DNA molecule of the present invention typically comprises a promoter operably or operatively linked to a DNA sequence encoding a 5' non-translated region, a DNA sequence of a plant intron, a structural sequence encoding a chloroplast transit peptide (CTP), a DNA coding sequence for a gene encoding improved herbicide tolerance, and a 3' non-translated region.

The 5' non-translated leader sequence can be derived from the promoter selected to express the heterologous DNA sequence, and can be specifically modified if desired so as to increase translation of mRNA. A 5' non-translated region can also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence which accompanies the promoter sequence. The leader sequence could also be derived from an unrelated promoter or coding sequence.

The 3' non-translated region of a plant operable recombinant DNA molecule contains a polyadenylation signal which functions in plants to cause the addition of adenylate nucleotides to the 3' end of the RNA. The 3' non-translated region can be obtained from various genes which are expressed in plant cells. The nopaline synthase 3' untranslated region (Fraley et al. 1983), the 3' untranslated region from pea ssRUBISCO (Coruzzi et al. 1994), the 3' untranslated region from soybean 7S seed storage protein gene (Schuler et al. 1982) and the pea small subunit of the pea ssRUBISCO gene are commonly used in this capacity. The 3' transcribed, non-translated regions containing the polyadenylate signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes are also suitable.

Examples of plant introns suitable for expression in monocots includes, for example, maize hsp70 intron, rice actin 1 intron, maize ADH 1 intron, *Arabidopsis* SSU intron, *Arabidopsis* EPSPS intron, petunia EPSPS intron and others known to those skilled in the art.

It may be particularly advantageous to direct the localization of proteins conferring herbicide tolerance to subcellular compartment, for example, to the mitochondrion, endoplasmic reticulum, vacuoles, chloroplast or other plastidic compartment. Proteins can be directed to the chloroplast by including at their amino-terminus a chloroplast transit peptide (CTP). Naturally occurring chloroplast targeted proteins, synthesized as larger precursor proteins containing an amino-terminal chloroplast targeting peptide directing the precursor to the chloroplast import machinery, have been previously identified and are well known in the art. Chloroplast targeting

peptides are generally cleaved by specific endoproteases located within the chloroplast organelle, thus releasing the targeted mature and preferably active enzyme from the precursor into the chloroplast melieu. Examples of sequences encoding peptides which are suitable for directing the targeting of the herbicide tolerance gene or transacylase gene product to the chloroplast or plastid of the plant cell include the petunia EPSPS CTP, the *Arabidopsis* EPSPS CTP2 and intron, and others known to those skilled in the art. Such targeting sequences provide for the desired expressed protein to be transferred to the cell structure in which it most effectively functions, or by transferring the desired expressed protein to areas of the cell in which cellular processes necessary for desired phenotypic function are concentrated. Chloroplast targeting peptides have been found to be particularly useful in the selection of glyphosate resistant plants (Barry et al., US Patent No. 5,463,175; Barry et al., US Patent No. 5,633,435). Glyphosate functions to kill the cell by inhibiting aromatic amino acid biosynthesis which takes place within the chloroplast. Therefor, concentrating the resistance gene product within the chloroplast provides increased resistance to the herbicide. The examples herein provide for a transacylase which is also targeted to or localized to and concentrated within the chloroplast. Specific examples of chloroplast targeting peptides are well known in the art and include the *Arabidopsis thaliana* ribulose bisphosphate carboxylase small subunit ats1A transit peptide, an *Arabidopsis thaliana* EPSPS transit peptide, and a *Zea maize* ribulose bisphosphate carboxylase small subunit transit peptide. One CTP that has functioned herein to localize heterologous proteins to the chloroplast was derived from the *Arabidopsis thaliana* ribulose bisphosphate carboxylase small subunit ats1A transit peptide. A polynucleotide sequence encoding a variant of this transit peptide used herein provides the native transit peptide amino acid sequence plus a reiteration of the transit peptide cleavage site, and has been shown herein to be useful for deploying active recombinant transacylase enzyme to the chloroplast (SEQ ID NO:9).

An alternative means for localizing plant operable herbicide tolerance or herbicide resistance genes to a chloroplast or plastid includes chloroplast or plastid transformation. Recombinant plants can be produced in which only the mitochondrial or chloroplast DNA has been altered to incorporate the molecules envisioned in this application. Promoters which function in chloroplasts have been known in the art (Hanley-Bowden et al., Trends in Biochemical Sciences 12:67-70, 1987). Methods and compositions for obtaining cells containing chloroplasts into which heterologous DNA has been inserted have been described, for

example by Daniell et al. (U.S. Pat. No. 5,693,507; 1997) and Maliga et al. (U.S. Pat. No. 5,451,513; 1995).

The accumulation of AMPA in plants can cause phytotoxic symptoms which are manifested phenotypically as chlorosis of the leaves, stunted growth, infertility, and death, although not all of these symptoms are evidenced in every species of plant. It has been discovered herein that enzymatic modification of the AMPA molecule by transacylation to produce N-acyl-AMPA provides a means for overcoming the phytotoxic effects of AMPA. A method for assaying the conversion of AMPA to N-acyl-AMPA involves providing [^{14}C] labeled AMPA as one substrate for the transacylase enzyme, and acyl-CoA as another substrate for the enzyme in an aqueous reaction volume, and separating the [^{14}C] labeled AMPA substrate from N-acyl-[^{14}C]-AMPA product by HPLC on an anion exchange column as described in the examples herein. Surprisingly, the transacylase enzyme has been shown to be capable of utilizing other acylated-CoA compounds as substrates for transacylating the AMPA substrate. In particular, propionyl-CoA was shown to be a particularly reactive substrate for the transacylation reaction *in vitro*, producing N-propionyl-[^{14}C]-AMPA. Larger acylated-CoA compounds, i.e. butyryl-CoA or methylmalonyl-CoA and other organic molecules covalently linked to CoA which have a carbon chain length greater than C_3 proved to be less effective in the transacylation reaction when using AMPA as the acyl-group recipient substrate. Notwithstanding this information, one skilled in the art would recognize that other transacylases which are substantially related by amino acid sequence homology to a PhnO or PhnO-like enzyme as characterized herein would have a similar substrate specificity in the AMPA transacylase reaction as compared to that encompassed by PhnO. These other enzymes too are conceptually within the scope and spirit of the invention described herein. For example, fatty acid biosynthesis is mediated by a wide range of acyl-CoA and acyl-carrier protein compounds which may be useful as substrates in transacylating phytotoxic compounds such as AMPA. A transacylase capable of AMPA transacylation using a fatty acid intermediate could conceivably provide plant protection by eliminating AMPA phytotoxicity. An enzyme such as PhnO, which is capable of transacylation, may be useful in detoxifying a wide range of toxic compounds which contain CP bonds and which additionally contain a CN linkage.

Methods and compositions for transforming a bacterium, a yeast or fungal cell, a plant cell, or an entire plant with one or more expression vectors comprising a *phn*O- or *phn*O-like

gene sequence are further aspects of this disclosure. A transgenic bacterium, yeast or fungal cell, plant cell, or plant derived from such a transformation process or the progeny and seeds from such a transgenic plant are also further embodiments of this invention.

Methods for transforming bacteria and yeast or fungal cells are well known in the art. Typically, means of transformation are similar to those well known means used to transform other bacteria, such as *E. coli*, or yeast, such as *Saccharomyces cerevisiae*. Methods for DNA transformation of plant cells include, but are not limited to *Agrobacterium*-mediated plant transformation, protoplast transformation, gene transfer into pollen, injection into reproductive organs, injection into immature embryos, plastid or chloroplast transformation, and particle bombardment. Each of these methods has distinct advantages and disadvantages. Thus, one particular method of introducing genes into a particular plant species may not be the most effective for another plant species, but it is well known by those skilled in the art which methods are useful for a particular plant species.

There are many methods for introducing transforming DNA segments into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods are believed to include virtually any method by which DNA can be introduced into a cell, such as by *Agrobacterium* infection, binary bacterial artificial chromosome (BIBAC) vectors (Hamilton et al., 1996), direct delivery of DNA such as, for example by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993), by desiccation/inhibition-mediated DNA uptake, by electroporation, by agitation with silicon carbide fibers, by acceleration of DNA coated particles, *etc.* In certain embodiments, acceleration methods are preferred and include, for example, microprojectile bombardment and the like.

Technology for introduction of DNA into cells is well-known to those of skill in the art. Four general methods for delivering a gene into cells have been described: (1) chemical methods (Graham and van der Eb, 1973; Zatloukal *et al.*, 1992); (2) physical methods such as microinjection (Capecchi, 1980), electroporation (Wong and Neuman, 1982; Fromm *et al.*, 1985; U.S. Patent No. 5,384,253) and the gene gun (Johnston and Tang, 1994; Fynan *et al.*, 1993; Luthra et al., 1997); (3) viral vectors (Clapp, 1993; Lu *et al.*, 1993; Eglitis and Anderson, 1988a; 1988b); and (4) receptor-mediated mechanisms (Curiel *et al.*, 1991; 1992; Wagner *et al.*, 1992)

Methods for transforming dicots, primarily by use *of Agrobacterium tumefaciens*, and obtaining transgenic plants have been published for cotton (U.S. Patent No. 5,004,863; U.S. Patent No. 5,159,135; U.S. Patent No. 5,518,908), soybean (U.S. Patent No. 5,569,834; U.S. Patent No. 5,416,011; McCabe *et al.* (1988); Christou *et al.* (1988)), *Brassica* (U.S. Patent No. 5,463,174), peanut (Cheng *et al.* (1996); De Kathen and Jabobsen (1990)).

Transformation of monocots using electroporation, particle bombardment, and *Agrobacterium* have also been reported. Transformation and plant regeneration have been achieved in asparagus (Bytebier *et al.* (1987)), barley (Wan and Lemaux (1994)), maize (Rhodes *et al.* (1988); Ishida *et al.* (1996); Gordon-Kamm *et al.* (1990); Fromm *et al.* (1990); Koziel *et al.* (1993); Armstrong *et al.* (1995), oat (Somers *et al.* (1992)), orchardgrass (Horn *et al.* (1988)), rice (Toriyama *et al.* (1988); Park *et al.* (1996); Abedinia *et al.* (1997); Zhang and Wu (1988); Zhang *et al.* (1988); Battraw and Hall (1990); Christou *et al.* (1991); Park *et al.* (1996)), rye (De la Pena *et al.* (1987)), sugar cane (Bower and Birch (1992)), tall fescue (Wang *et al.* (1992)), and wheat (Vasil *et al.* (1992); Weeks *et al.* (1993)). Techniques for monocot transformation and plant regeneration are also discussed in Davey *et al.* (1986).

Recombinant plants could also be produced in which only the mitochondrial or chloroplast DNA has been altered to incorporate the molecules envisioned in this application. Promoters which function in chloroplasts have been known in the art (Handley-Bowden et al., Trends in Biochemical Sciences 12:67-70, 1987). Methods and compositions for obtaining cells containing chloroplasts into which heterologous DNA has been inserted has been described by Daniell et al., U.S. Pat. No. 5,693,507 (1997) and Maliga et al. (U.S. Pat. No. 5,451,513; 1995). Recombinant plants which have been transformed using heterologous DNA, altering both nuclear and chloroplast or plastidic genomes is also within the scope of this invention.

The present invention discloses DNA constructs comprising polynucleotide sequences encoding AMPA-transacylase. Methods for identifying and isolating heterologous genes encoding peptides which function in N-acylation of AMPA are disclosed herein. Methods for the construction and expression of synthetic genes in plants are well known by those of skill in the art and are described in detail in U. S. Patent No. 5,500,365, and in monocotyledonous plants in particular in U.S. Patent No. 5,689,052. The present invention contemplates the use of AMPA acyltransferase genes alone or in combination with genes encoding GOX mediated glyphosate degradation enzymes in the transformation of both monocotyledonous and dicotyledonous

plants. To potentiate the expression of these genes, the present invention provides DNA constructs comprising polynucleotide sequences encoding these types of proteins which are localized to the plant cell cytoplasm as well as sequences encoding plastid targeting peptides positioned upstream of the polynucleotide sequences encoding the AMPA transacylase and/or GOX proteins.

In one aspect, nucleotide sequence information provided by the invention allows for the preparation of relatively short DNA sequences having the ability to specifically hybridize to gene sequences of the selected polynucleotides disclosed herein. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of selected polynucleotide sequences encoding AMPA transacylase polypeptides, *e.g.*, sequences such as are shown in SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3. Such nucleic acid probes may also be prepared based on a consideration of selected polynucleotide sequences encoding a plastid targeting peptide, such as those shown in SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, and SEQ ID NO:14. The ability of such nucleic acid probes to specifically hybridize to a gene sequence encoding an AMPA transacylase polypeptide or a plastid targeting peptide sequence lends to them particular utility in a variety of embodiments. Most importantly, the probes may be used in a variety of assays for detecting the presence of complementary sequences in a given sample.

In certain embodiments, it is advantageous to use oligonucleotide primers. The sequence of such primers is designed using a polynucleotide of the present invention for use in detecting, amplifying or mutating a defined sequence of a AMPA transacylase gene from any suitable organism using PCR™ technology. The process may also be used to detect, amplify or mutate a defined sequence of the polynucleotide encoding a plastid targeting peptide. Segments of genes related to the polynucleotides encoding the AMPA transacylase polypeptides and plastid targeting peptides of the present invention may also be amplified by PCR™ using such primers.

To provide certain of the advantages in accordance with the present invention, a preferred nucleic acid sequence employed for hybridization studies or assays includes sequences that are substantially complementary to at least a length of 14 to 30 or so consecutive nucleotides of a polynucleotide sequence flanking, in cis with, or encoding an AMPA transacylase, such as that shown in SEQ ID NO:5 or SEQ ID NO:6, or sequences that are substantially complementary to at least a length of 14 to 30 or so consecutive nucleotides of a sequence encoding a plastid targeting peptide. By "substantially complimentary", it is meant that a polynucleotide is

preferably about 70% complimentary, or more preferably about 80% complimentary, or even more preferably about 90% complimentary, or most preferably about 99-100% complimentary in sequence to a target polynucleotide sequence.

A size of at least 14 nucleotides in length helps to ensure that the fragment will be of sufficient length to form a duplex molecule that is both stable and selective. Molecules having complementary sequences over segments greater than 14 bases in length are generally preferred. In order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained, one will generally prefer to design nucleic acid molecules having gene-complementary sequences of 14 to 20 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, such as phosphoramidite chemistries for example; by application of nucleic acid reproduction technology, such as the PCR™ technology of U. S. Patents 4,683,195, and 4,683,202 (each specifically incorporated herein by reference); or by excising selected DNA fragments from recombinant plasmids containing appropriate inserts and suitable restriction sites.

The present invention also contemplates an expression vector comprising a polynucleotide of the present invention. Thus, in one embodiment an expression vector is an isolated and purified DNA molecule comprising a promoter operably linked to a coding region that encodes a polypeptide of the present invention, which coding region is operatively linked to a transcription-terminating region, whereby the promoter drives the transcription of the coding region. The coding region may include a segment or sequence encoding a AMPA transacylase and a segment or sequence encoding a plastid targeting peptide. The DNA molecule comprising the expression vector may also contain a plant functional intron, and may also contain other plant functional elements such as sequences encoding untranslated sequences (UTL's) and sequences which act as enhancers of transcription or translation.

As used herein, the terms "operatively linked" or "operably linked" mean that a sequence which functions as a promoter is connected or linked to a coding region in such a way that the transcription of that coding region is controlled and regulated by that promoter. Means for operatively linking a promoter to a coding region to regulate both upstream and downstream are well known in the art.

Preferred plant transformation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens*, as well as those disclosed, *e.g.*, by Herrera-Estrella (1983), Bevan (1983), Klee (1985) and Eur. Pat. Appl. No. EP 0120516 (each specifically incorporated herein by reference). In addition, plant preferred transformation vectors directed to chloroplast or plastid transformation include those disclosed in U.S. Pat. No. 5,693,507 (1997), U.S. Pat. No. 5,451,513 (1995), McBride et al. (1995), Staub et al. (1995a), Staub et al. (1995b), and WO 95/24492 (each specifically incorporated herein by reference).

Where an expression vector of the present invention is to be used to transform a plant, a promoter is selected that has the ability to drive expression in that particular species of plant. Promoters that function in different plant species are also well known in the art. Promoters useful in expressing the polypeptide in plants are those which are inducible, viral, synthetic, or constitutive as described (Odell *et al.*, 1985), and/or temporally regulated, spatially regulated, and spatio-temporally regulated. Preferred promoters include the enhanced CaMV35S promoters, and the FMV35S promoter.

The expression of a gene which exists in double-stranded DNA form localized to the plant nuclear genome involves transcription of messenger RNA (mRNA) from the coding strand of the DNA by an RNA polymerase enzyme, and the subsequent processing of the mRNA primary transcript inside the nucleus. Genes expressed from within a chloroplast or plastid also produce an mRNA transcript which is not processed further prior to translation. In any event, transcription of DNA into mRNA is regulated by a region of DNA referred to as the "promoter". The DNA comprising the promoter is represented by a sequence of bases that signals RNA polymerase to associate with the DNA and to initiate the transcription of mRNA using one of the DNA strands as a template to make a corresponding strand of RNA. The particular promoter selected should be capable of causing sufficient expression of an AMPA acyltransferase enzyme coding sequence to result in the production of an herbicide tolerance effective or herbicide resistance effective amount of the transacylase protein localized to the desired intracellular location.

Structural genes can be driven by a variety of promoters in plant tissues. Promoters can be near-constitutive (*i.e.* they drive transcription of the transgene in all tissue), such as the CaMV35S promoter, or tissue-specific or developmentally specific promoters affecting dicots or monocots. Where the promoter is a near-constitutive promoter such as CaMV35S or FMV35S,

increases in polypeptide expression are found in a variety of transformed plant tissues and most plant organs (*e.g.*, callus, leaf, seed, stem, meristem, flower, and root). Enhanced or duplicate versions of the CaMV35S and FMV35S promoters are particularly useful in the practice of this invention (Kay *et al.*, 1987; Rogers, U. S. Patent 5,378,619).

Those skilled in the art will recognize that there are a number of promoters which are active in plant cells, and have been described in the literature. Such promoters may be obtained from plants or plant viruses and include, but are not limited to, the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on tumor-inducing plasmids of *A. tumefaciens*), the cauliflower mosaic virus (CaMV) 19S and 35S promoters, the light-inducible promoter from the small subunit of ribulose 1,5-bisphosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide), the rice *Act1* promoter and the Figwort Mosaic Virus (FMV) 35S promoter. All of these promoters have been used to create various types of DNA constructs which have been expressed in plants (see *e.g.*, McElroy *et al.*, 1990, U. S. Patent 5,463,175).

In addition, it may also be preferred to bring about expression of genes such as an AMPA acyltransferase which improve herbicide tolerance or herbicide resistance in specific tissues of a plant by using plant integrating vectors containing a tissue-specific promoter. Specific target tissues may include the leaf, stem, root, tuber, seed, fruit, *etc.*, and the promoter chosen should have the desired tissue and developmental specificity. Therefore, promoter function should be optimized by selecting a promoter with the desired tissue expression capabilities and approximate promoter strength, and selecting a transformant which produces the desired transacylase activity in the target tissues. This selection approach from the pool of transformants is routinely employed in expression of heterologous structural genes in plants since there is variation between transformants containing the same heterologous gene due to the site of gene insertion within the plant genome (commonly referred to as "position effect"). In addition to promoters which are known to cause transcription (constitutive or tissue-specific) of DNA in plant cells, other promoters may be identified for use in the current invention by screening a plant cDNA library for genes which are selectively or preferably expressed in the target tissues, then determining the promoter regions. Chloroplast or plastid functional promoters are known in the art (Hanley-Bowden et al., Daniell et al., Maliga et al.).

Other exemplary tissue-specific promoters are corn sucrose synthetase 1 (Yang *et al.*, 1990), corn alcohol dehydrogenase 1 (Vogel *et al.*, 1989), corn light harvesting complex (Simpson, 1986), corn heat shock protein (Odell *et al.*, 1985), pea small subunit RuBP carboxylase (Poulsen *et al.*, 1986; Cashmore *et al.*, 1983), Ti plasmid mannopine synthase (McBride and Summerfelt, 1989), Ti plasmid nopaline synthase (Langridge *et al.*, 1989), petunia chalcone isomerase (Van Tunen *et al.*, 1988), bean glycine rich protein 1 (Keller *et al.*, 1989), CaMV 35s transcript (Odell *et al.*, 1985) and Potato patatin (Wenzler *et al.*, 1989) promoters. Preferred promoters are the cauliflower mosaic virus (CaMV 35S) promoter and the S-E9 small subunit RuBP carboxylase promoter.

The promoters used in the DNA constructs of the present invention may be modified, if desired, to affect their control characteristics. For example, the CaMV35S promoter may be ligated to the portion of the ssRUBISCO gene that represses the expression of ssRUBISCO in the absence of light, to create a promoter which is active in leaves but not in roots.. For purposes of this description, the phrase "CaMV35S" promoter thus includes variations of CaMV35S promoter, *e.g.*, promoters derived by means of ligation with operator regions, random or controlled mutagenesis, *etc.* Furthermore, the promoters may be altered to contain multiple "enhancer sequences" to assist in elevating gene expression. Examples of such enhancer sequences have been reported by Kay *et al.* (1987). Chloroplast or plastid specific promoters are known in the art (Daniell et al., US Pat. No. 5,693,507; herein incorporated by reference). Promoters obtainable from chloroplast genes, for example, such as the *psb*A gene from spinach or pea, the *rbc*L and *atp*B promoter regions from maize, and rRNA promoters. Any chloroplast or plastid operable promoter is within the scope of the present invention.

A transgenic plant of the present invention produced from a plant cell transformed with a tissue specific promoter can be crossed with a second transgenic plant developed from a plant cell transformed with a different tissue specific promoter to produce a hybrid transgenic plant that shows the effects of transformation in more than one specific tissue.

The RNA produced by a DNA construct of the present invention may also contain a 5' non-translated leader sequence (5'UTL). This sequence can be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to

constructs wherein the non-translated region is derived from the 5′ non-translated sequence that accompanies the promoter sequence. One plant gene leader sequence for use in the present invention is the petunia heat shock protein 70 (hsp70) leader (Winter *et al.*, 1988).

5' UTL's are capable of regulating gene expression when localized to the DNA sequence between the transcription initiation site and the start of the coding sequence. Compilations of leader sequences have been made to predict optimum or sub-optimum sequences and generate "consensus" and preferred leader sequences (Joshi, 1987). Preferred leader sequences are contemplated to include those which comprise sequences predicted to direct optimum expression of the linked structural gene, *i.e.* to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure. Sequences that are derived from genes that are highly expressed in plants, and in maize in particular, will be most preferred. One particularly useful leader may be the petunia HSP70 leader.

In accordance with the present invention, expression vectors designed to specifically potentiate the expression of the polypeptide in the transformed plant may include certain regions encoding plastid or chloroplast targeting peptides, herein abbreviated in various forms as CTP, CTP1, CTP2, etc., each representing a different or variant targeting peptide sequence. These regions allow for the cellular processes involved in transcription, translation and expression of the encoded protein to be fully exploited when associated with certain GOX or AMPA transacylase protein sequences. Such targeting peptides function in a variety of ways, such as for example, by transferring the expressed protein to the cell structure in which it most effectively operates, or by transferring the expressed protein to areas of the cell in which cellular processes necessary for expression are concentrated. The use of CTP's may also increase the frequency of recovery of morphologically normal plants, and the frequency at which transgenic plants may be recovered.

Chloroplast targeting peptides have been found particularly useful in the glyphosate resistant selectable marker system. In this system, plants transformed to express a protein conferring glyphosate resistance are transformed along with a CTP that targets the peptide to the plant cell's chloroplasts. Glyphosate inhibits the shikimic acid pathway which leads to the biosynthesis of aromatic compounds including amino acids and vitamins. Specifically,

glyphosate inhibits the conversion of phosphoenolpyruvic acid and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid by inhibiting the enzyme 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSP synthase or EPSPS). Introduction of a transgene encoding EPSPS allows the plant cell to resist the effects of glyphosate, especially when the transgene encodes a glyphosate insensitive EPSPS enzyme. Thus, as the herbicide glyphosate functions to kill the cell by interrupting aromatic amino acid biosynthesis, particularly in the cell's chloroplast, the CTP allows increased resistance to the herbicide by concentrating what glyphosate resistance enzyme the cell expresses in the chloroplast, *i.e.* in the target organelle of the cell. Exemplary herbicide resistance enzymes include EPSPS and glyphosate oxido-reductase (GOX) genes (see Comai, 1985, U.S. Patent No. 4,535,060, specifically incorporated herein by reference in its entirety).

CTPs can target proteins to chloroplasts and other plastids. For example, the target organelle may be the amyloplast. Preferred CTP's of the present invention include those targeting both chloroplasts as well as other plastids. Specific examples of preferred CTP's include the maize RUBISCO SSU protein CTP, and functionally related peptides such as the *Arabidopsis thaliana* RUBISCO small subunit CTP and the *Arabidopsis thaliana* EPSPS CTP. These CTP's are exemplified by the polynucleotide and amino acid sequences shown in SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, and SEQ ID NO:14 respectively.

Recombinant plants, cells, seeds, and other plant tissues could also be produced in which only the mitochondrial or chloroplast DNA has been altered to incorporate the molecules envisioned in this application. Promoters which function in chloroplasts have been known in the art (Hanley-Bowden et al., Trends in Biochemical Sciences **12**:67-70, 1987). Methods and compositions for obtaining cells containing chloroplasts into which heterologous DNA has been inserted has been described in U.S. Pat. No. 5,693,507 (1997). McBride et al. (WO 95/24492) disclose localization and expression of genes encoding Cry1A δ-endotoxin protein in tobacco plant chloroplast genomes.

An exemplary embodiment of the invention involves the plastid or chloroplast targeting or plastid or chloroplast localization of genes encoding enzymes or proteins conferring herbicide tolerance or herbicide resistance in plants. Plastid or chloroplast targeting sequences have been isolated from numerous nuclear encoded plant genes and have been shown to direct importation of cytoplasmically synthesized proteins into plastids or chloroplasts (reviewed in Keegstra and

Olsen, 1989). A variety of plastid targeting sequences, well known in the art, including but not limited to ADPGPP, EPSP synthase, or ssRUBISCO, may be utilized in practicing this invention. In addition, plastidic targeting sequences (peptide and nucleic acid) for monocotyledonous crops may consist of a genomic coding fragment containing an intron sequence as well as a duplicated proteolytic cleavage site in the encoded plastidic targeting sequences.

The preferred CTP sequence for dicotyledonous crops is referred to herein as (SEQ ID NO:9), and consists of a genomic coding fragment containing the chloroplast targeting peptide sequence from the EPSP synthase gene of *Arabidopsis thaliana* in which the transit peptide cleavage site of the pea ssRUBISCO CTP replaces the native EPSP synthase CTP cleavage site (Klee *et al.*, 1987).

For optimized expression in monocotyledonous plants, an intron may also be included in the DNA expression construct. Such an intron is typically placed near the 5′ end of the mRNA in untranslated sequence. This intron could be obtained from, but not limited to, a set of introns consisting of the maize heat shock protein (HSP) 70 intron (U. S. Patent No. 5,424,412; 1995), the rice *Act1* intron (McElroy *et al.*, 1990), the Adh intron 1 (Callis *et al.*, 1987), or the sucrose synthase intron (Vasil *et al.*, 1989).

The 3′ non-translated region of the genes of the present invention which are localized to the plant nuclear genome also contain a polyadenylation signal which functions in plants to cause the addition of adenylate nucleotides to the 3′ end of the mRNA. RNA polymerase transcribes a nuclear genome coding DNA sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA). Examples of preferred 3′ regions are (1) the 3′ transcribed, non-translated regions containing the polyadenylation signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene and (2) the 3′ ends of plant genes such as the pea ribulose-1,5-bisphosphate carboxylase small subunit gene, designated herein as E9 (Fischhoff *et al.*, 1987). Constructs will typically include the gene of interest along with a 3′ end DNA sequence that acts as a signal to terminate transcription and, in constructs intended for nuclear genome expression, allow for the poly-adenylation of the resultant mRNA. The most

preferred 3' elements are contemplated to be those from the nopaline synthase gene of *A. tumefaciens* (*nos* 3'end) (Bevan *et al.*, 1983), the terminator for the T7 transcript from the octopine synthase gene of *A. tumefaciens*, and the 3' end of the protease inhibitor I or II genes from potato or tomato. Regulatory elements such as TMV Ω element (Gallie, *et al.*, 1989), may further be included where desired.

According to the present invention and as noted above, chloroplast or plastid localized genes encoding enzymes conferring herbicide tolerance or herbicide resistance characteristics to plants do not require sequences which confer transcription termination and polyadenylation signals, but instead may only require transcription termination information at the 3' end of the gene. For coding sequences introduced into a chloroplast or plastid, or into a chloroplast or plastid genome, mRNA transcription termination is similar to methods well known in the bacterial gene expression art. For example, either in a polycistronic or a monocistronic sequence, transcription can be terminated by stem and loop structures or by structures similar to *rho* dependent sequences.

Transcription enhancers or duplications of enhancers could be used to increase expression. These enhancers often are found 5' to the start of transcription in a promoter that functions in eukaryotic cells, but can often be inserted in the forward or reverse orientation 5' or 3' to the coding sequence. Examples of enhancers include elements from the CaMV 35S promoter, octopine synthase genes (Ellis *et al.*, 1987), the rice actin gene, and promoter from non-plant eukaryotes (*e.g.*, yeast; Ma *et al.*, 1988).

In certain embodiments of the invention, the use of internal ribosome binding sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5 methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Samow, 1991). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message.

Any heterologous open reading frame can be linked to IRES elements. This includes genes for secreted proteins, multi-subunit proteins, encoded by independent genes, intracellular or membrane-bound proteins and selectable markers. In this way, expression of several proteins can be simultaneously engineered into a cell with a single construct and a single selectable marker.

Constructs intended for expression from within a chloroplast or plastid utilizing chloroplast or plastid specific transcriptional and translational machinery can contain either mono- or polycistronic sequences.

The choice of which expression vector and ultimately to which promoter a polypeptide coding region is operatively linked depends directly on the functional properties desired, *e.g.*, the location and timing of protein expression, and the host cell to be transformed. These are well known limitations inherent in the art of constructing recombinant DNA molecules. However, a vector useful in practicing the present invention is capable of directing the expression of the polypeptide coding region to which it is operatively linked.

Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *A. tumefaciens* described (Rogers *et al.*, 1987). However, several other plant integrating vector systems are known to function in plants including pCaMVCN transfer control vector described (Fromm *et al.*, 1985). pCaMVCN (available from Pharmacia, Piscataway, NJ) includes the CaMV35S promoter.

In preferred embodiments, the vector used to express the polypeptide includes a selection marker that is effective in a plant cell, preferably a drug resistance selection marker. One preferred drug resistance marker is the gene whose expression results in kanamycin resistance; *i.e.* the chimeric gene containing the nopaline synthase promoter, Tn*5* neomycin phosphotransferase II (*npt*II) and nopaline synthase 3′ non-translated region described (Rogers *et al.*, 1988).

Means for preparing expression vectors are well known in the art. Expression (transformation) vectors used to transform plants and methods of making those vectors are described in U. S. Patents 4,971,908, 4,940,835, 4,769,061 and 4,757,011 (each of which is specifically incorporated herein by reference). Those vectors can be modified to include a coding sequence in accordance with the present invention.

A variety of methods have been developed to operatively link DNA to vectors *via* complementary cohesive termini or blunt ends. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted and to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

A coding region that encodes a polypeptide having the ability to confer enhanced herbicide resistance enzymatic activity to a cell is preferably a polynucleotide encoding an AMPA transacylase or a functional equivalent, alone or in combination, with a gene encoding a GOX enzyme or a functional equivalent of GOX. In accordance with such embodiments, a coding region comprising the DNA sequence of SEQ ID NO:3, SEQ ID NO:7, or SEQ ID NO:19 is also preferred.

Specific genes encoding AMPA transacylase that have been shown to successfully transform plants in conjunction with plastid targeting peptide-encoding genes, to express the AMPA transacylase at sufficient herbicidally protective levels are those genes comprised within the plasmid vectors. Preferred plasmids containing plastid targeting sequences include pMON17261, pMON10151, pMON10149, pMON32570, pMON32571, pMON32572, pMON32573, pMON32926, pMON32931, pMON32932, pMON32936, pMON32938, pMON32946, pMON32947, pMON32948, and pMON32950. These plasmids contain polynucleotide sequences which encode targeting sequences as shown in SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:14. Expression cassettes comprising plant operable promoters linked to coding sequences, some with and some without 5' untranslated sequences and/or intron sequences, wherein the coding sequences contain at least an AMPA transacylase or transacetylase, linked to plant operable termination sequences are disclosed in particular as set forth in SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29, and SEQ ID NO:31.

The work described herein has identified methods of potentiating *in planta* expression of an AMPA transacylase, which confer protection from glyphosate and related herbicides to plants when incorporated into the nuclear, plastid, or chloroplast genome of susceptible plants which also express a GOX or similar gene. U. S. Patent 5,500,365 (specifically incorporated herein by reference) describes a method for synthesizing plant genes to optimize the expression level of the protein for which the synthesized gene encodes. This method relates to the modification of the structural gene sequences of the exogenous transgene, to make them more "plant-like" and

therefore more likely to be translated and expressed by the plant. A similar method for enhanced expression of transgenes, preferably in monocotyledonous plants, is disclosed in U. S. Patent 5,689,052 (specifically incorporated herein by reference). Agronomic, horticultural, ornamental, and other economically or commercially useful plants can be made in accordance with the methods described herein.

Such plants may co-express the AMPA transacylase gene and/or a GOX gene along with other antifungal, antibacterial, or antiviral pathogenesis-related peptides, polypeptides, or proteins; insecticidal proteins; other proteins conferring herbicide resistance; and proteins involved in improving the quality of plant products or agronomic performance of plants. Simultaneous co-expression of multiple heterologous proteins in plants is advantageous in that it can exploits more than one mode of action to control plant damage or improve the quality of the plant or products produced by the plants metabolism.

It is contemplated that introduction of large DNA sequences comprising more than one gene may be desirable. Introduction of such sequences may be facilitated by use of bacterial or yeast artificial chromosomes (BACs or YACs, respectively), or even plant artificial chromosomes. For example, the use of BACs for *Agrobacterium*-mediated transformation was disclosed by Hamilton *et al.* (1996).

Ultimately, the most desirable DNA sequences for introduction into a monocot genome may be homologous genes or gene families which encode a desired trait (for example, increased yield), and which are introduced under the control of novel promoters or enhancers, *etc.*, or perhaps even homologous or tissue specific (*e.g.*, root-collar/sheath-, whorl-, stalk-, earshank-, kernel- or leaf-specific) promoters or control elements. Indeed, it is envisioned that a particular use of the present invention may be the production of transformants comprising a transgene which is targeted in a tissue-specific manner. For example, herbicide resistance or herbicide tolerance genes may be expressed specifically or specifically regulated in a negative manner in the plants reproductive tissues which can provide a means for enhancing herbicide tolerance or sensitivity to those tissues. Such regulatory control means can provide methods for regulating the escape of transgenes into the environment or for controlling the illicit use of proprietary or licensed intellectual or commercialized property.

Vectors for use in tissue-specific targeting of gene expression in transgenic plants typically will include tissue-specific promoters and also may include other tissue-specific control

elements such as enhancer sequences. Promoters which direct specific or enhanced expression in certain plant tissues will be known to those of skill in the art in light of the present disclosure.

It also is contemplated that tissue specific expression may be functionally accomplished by introducing a constitutively expressed gene (all tissues) in combination with an antisense gene that is expressed only in those tissues where the gene product is not desired. For example, a gene coding for the AMPA transacylase from *E. coli* may be introduced such that it is expressed in all tissues using the 35S promoter from Cauliflower Mosaic Virus. Alternatively, a rice actin promoter or a histone promoter from a dicot or monocot species also could be used for constitutive expression of a gene. Furthermore, it is contemplated that promoters combining elements from more than one promoter may be useful. For example, U. S. Patent 5,491,288 discloses combining a Cauliflower Mosaic Virus promoter with a histone promoter. Therefore, expression of an antisense transcript of the AMPA transacylase gene in a maize kernel, using for example a zein promoter, would prevent accumulation of the transacylase in seed. Thus, in a plant expressing both GOX and the transacylase, application of glyphosate herbicide would result in seed tissues which fail to mature. Conversely, antisense suppression of the GOX gene would effectuate the same result. Preferably, suppression of the transacylase in specific tissues would be more advantageous, particularly where specific tissues have demonstrated an intolerance to AMPA or related compounds. It is specifically contemplated by the inventor that a similar strategy could be used with the instant invention to direct expression of a screenable or selectable marker in seed tissue.

Alternatively, one may wish to obtain novel tissue-specific promoter sequences for use in accordance with the present invention. To achieve this, one may first isolate cDNA clones from the tissue concerned and identify those clones which are expressed specifically in that tissue, for example, using Northern blotting. Ideally, one would like to identify a gene that is not present in a high copy number, but which gene product is relatively abundant in specific tissues. The promoter and control elements of corresponding genomic clones may this be localized using the techniques of molecular biology known to those of skill in the art.

It is contemplated that expression of some genes in transgenic plants will be desired only under specified conditions. For example, it is proposed that expression of certain genes that confer resistance to environmentally stress factors such as drought will be desired only under actual stress conditions. It further is contemplated that expression of such genes throughout a

plants development may have detrimental effects. It is known that a large number of genes exist that respond to the environment. For example, expression of some genes such as *rbcS*, encoding the small subunit of ribulose bisphosphate carboxylase, is regulated by light as mediated through phytochrome. Other genes are induced by secondary stimuli. For example, synthesis of abscisic acid (ABA) is induced by certain environmental factors, including but not limited to water stress. A number of genes have been shown to be induced by ABA (Skriver and Mundy, 1990). It also is expected that expression of genes conferring resistance to applications of herbicides would be desired only under conditions in which herbicide is actually present. Therefore, for some desired traits, inducible expression of genes in transgenic plants will be desired.

It is proposed that, in some embodiments of the present invention, expression of a gene in a transgenic plant will be desired only in a certain time period during the development of the plant. Developmental timing frequently is correlated with tissue specific gene expression. For example expression of zein storage proteins is initiated in the endosperm about 15 days after pollination.

It also is contemplated that it may be useful to specifically target DNA insertion within a cell. For example, it may be useful to target introduced DNA to the nucleus, and in particular into a precise position within one of the plant chromosomes in order to achieve site specific integration. For example, it would be useful to have a gene introduced through transformation which acts to replace an existing gene in the cell, or to complement a gene which is not functional or present at all.

A plant transformed with an expression vector of the present invention is also contemplated. A transgenic plant derived from such a transformed or transgenic cell is also contemplated. Those skilled in the art will recognize that a chimeric plant gene containing a structural coding sequence of the present invention can be inserted into the genome of a plant by methods well known in the art. Such methods for DNA transformation of plant cells include *Agrobacterium*-mediated plant transformation, the use of liposomes, transformation using viruses or pollen, electroporation, protoplast transformation, gene transfer into pollen, injection into reproductive organs, injection into immature embryos and particle bombardment. Each of these methods has distinct advantages and disadvantages. Thus, one particular method of introducing genes into a particular plant strain may not necessarily be the most effective for another plant strain, but it is well known which methods are useful for a particular plant strain.

There are many methods for introducing transforming DNA segments into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods are believed to include virtually any method by which DNA can be introduced into a cell, such as infection by *A. tumefaciens* and related *Agrobacterium* strains, direct delivery of DNA such as, for example, by PEG-mediated transformation of protoplasts (Omirulleh *et al.*, 1993), by desiccation/inhibition-mediated DNA uptake, by electroporation, by agitation with silicon carbide fibers, by acceleration of DNA coated particles, *etc.* In certain embodiments, acceleration methods are preferred and include, for example, microprojectile bombardment and the like.

Technology for introduction of DNA into cells is well-known to those of skill in the art. Four general methods for delivering a gene into cells have been described: (1) chemical methods (Graham and van der Eb, 1973); (2) physical methods such as microinjection (Capecchi, 1980), electroporation (Wong and Neumann, 1982; Fromm *et al.*, 1985) and the gene gun (Johnston and Tang, 1994; Fynan *et al.*, 1993); (3) viral vectors (Clapp, 1993; Lu *et al.*, 1993; Eglitis and Anderson, 1988a; 1988b); and (4) receptor-mediated mechanisms (Curiel *et al.*, 1991; 1992; Wagner *et al.*, 1992).

The application of brief, high-voltage electric pulses to a variety of animal and plant cells leads to the formation of nanometer-sized pores in the plasma membrane. DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of the redistribution of membrane components that accompanies closure of the pores. Electroporation can be extremely efficient and can be used both for transient expression of cloned genes and for establishment of cell lines that carry integrated copies of the gene of interest. Electroporation, in contrast to calcium phosphate-mediated transfection and protoplast fusion, frequently gives rise to cell lines that carry one, or at most a few, integrated copies of the foreign DNA.

The introduction of DNA by means of electroporation is well-known to those of skill in the art. To effect transformation by electroporation, one may employ either friable tissues such as a suspension culture of cells, or embryogenic callus, or alternatively, one may transform immature embryos or other organized tissues directly. One would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner, rendering the cells more susceptible to transformation. Such cells would then be recipient to DNA transfer by electroporation, which

may be carried out at this stage, and transformed cells then identified by a suitable selection or screening protocol dependent on the nature of the newly incorporated DNA.

A further advantageous method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like. Using these particles, DNA is carried through the cell wall and into the cytoplasm on the surface of small metal particles as described (Klein *et al.*, 1987; Klein *et al.*, 1988; Kawata *et al.*, 1988). The metal particles penetrate through several layers of cells and thus allow the transformation of cells within tissue explants. The microprojectile bombardment method is preferred for the identification of chloroplast or plastid directed transformation events.

An advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly stably transforming plant cells, is that neither the isolation of protoplasts (Cristou *et al.*, 1988) nor the susceptibility to *Agrobacterium* infection is required. An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is a Biolistics Particle Delivery System, which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with the plant cultured cells in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. It is believed that a screen intervening between the projectile apparatus and the cells to be bombarded reduces the size of projectiles aggregate and may contribute to a higher frequency of transformation by reducing damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the microprojectile stopping plate. If desired, one or more screens are also positioned between the acceleration device and the cells to be bombarded. Through the use of techniques set forth here-in one may obtain up to 1000 or more foci of cells transiently expressing a marker gene. The number of cells in a focus which express the exogenous gene product 48 hours post-bombardment often range from 1 to 10 and average 1 to 3.

In bombardment transformation, one may optimize the pre-bombardment culturing conditions and the bombardment parameters to yield the maximum numbers of stable transformants. Both the physical and biological parameters for bombardment are important in this technology. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the flight and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmids. It is believed that pre-bombardment manipulations are especially important for successful transformation of immature plant embryos.

Accordingly, it is contemplated that one may desire to adjust various of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance, and helium pressure. One may also minimize the trauma reduction factors (TRFs) by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. The execution of other routine adjustments will be known to those of skill in the art in light of the present disclosure.

The methods of particle-mediated transformation is well-known to those of skill in the art. U. S. Patent 5,015,580 (specifically incorporated herein by reference) describes the transformation of soybeans using such a technique.

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described (Fraley *et al.*, 1985; Rogers *et al.*, 1987). The genetic engineering of cotton plants using *Agrobacterium*-mediated transfer is described in U. S. Patent 5,004,863 (specifically incorporated herein by reference); like transformation of lettuce plants is described in U. S. Patent 5,349,124 (specifically incorporated herein by reference); and the *Agrobacterium*-mediated transformation of soybean is described in U. S. Patent 5,416,011

(specifically incorporated herein by reference). Further, the integration of the Ti-DNA is a relatively precise process resulting in few rearrangements. The region of DNA to be transferred is defined by the border sequences, and intervening DNA is usually inserted into the plant genome as described (Spielmann *et al.*, 1986; Jorgensen *et al.*, 1987).

Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee *et al.*, 1985). Moreover, recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate construction of vectors capable of expressing various polypeptide coding genes. The vectors described (Rogers *et al.*, 1987), have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant varieties where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

Agrobacterium-mediated transformation of leaf disks and other tissues such as cotyledons and hypocotyls appears to be limited to plants that *Agrobacterium* naturally infects. *Agrobacterium*-mediated transformation is most efficient in dicotyledonous plants. Few monocots appear to be natural hosts for *Agrobacterium*, although transgenic plants have been produced in asparagus using *Agrobacterium* vectors as described (Bytebier *et al.*, 1987). Other monocots recently have also been transformed with *Agrobacterium*. Included in this group are corn (Ishida *et al.*) and rice (Cheng *et al.*).

A transgenic plant formed using *Agrobacterium* transformation methods typically contains a single gene on one chromosome. Such transgenic plants can be referred to as being heterozygous for the added gene. However, inasmuch as use of the word "heterozygous" usually implies the presence of a complementary gene at the same locus of the second chromosome of a pair of chromosomes, and there is no such gene in a plant containing one added gene as here, it is believed that a more accurate name for such a plant is an independent segregant, because the added, exogenous gene segregates independently during mitosis and meiosis.

An independent segregant may be preferred when the plant is commercialized as a hybrid, such as corn. In this case, an independent segregant containing the gene is crossed with another plant, to form a hybrid plant that is heterozygous for the gene of interest.

An alternate preference is for a transgenic plant that is homozygous for the added structural gene; *i.e.* a transgenic plant that contains two added genes, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) an independent segregant transgenic plant that contains a single added gene, germinating some of the seed produced and analyzing the resulting plants produced for gene of interest activity and mendelian inheritance indicating homozygosity relative to a control (native, non-transgenic) or an independent segregant transgenic plant.

Two different transgenic plants can be mated to produce offspring that contain two independently segregating added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes that encode a polypeptide of interest. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated.

Transformation of plant protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see *e.g.*, Potrykus *et al.*, 1985; Lorz *et al.*, 1985; Fromm *et al.*, 1985; Uchimiya *et al.*, 1986; Callis *et al.*, 1987; Marcotte *et al.*, 1988).

Application of these systems to different plant germplasm depends upon the ability to regenerate that particular plant variety from protoplasts. Illustrative methods for the regeneration of cereals from protoplasts are described (see, *e.g.*, Fujimura *et al.*, 1985; Toriyama *et al.*, 1986; Yamada *et al.*, 1986; Abdullah *et al.*, 1986).

To transform plant germplasm that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described (Vasil, 1988).

Unmodified bacterial genes are often poorly expressed in transgenic plant cells. Plant codon usage more closely resembles that of humans and other higher organisms than unicellular organisms, such as bacteria. Several reports have disclosed methods for improving expression of recombinant genes in plants (Murray *et al.*, 1989; Diehn *et al.*, 1996; Iannacone *et al.*, 1997;

Rouwendal *et al.*, 1997; Futterer *et al.*, 1997; and Futterer and Hohn, 1996). These reports disclose various methods for engineering coding sequences to represent sequences which are more efficiently translated based on plant codon frequency tables, improvements in codon third base position bias, using recombinant sequences which avoid suspect polyadenylation or A/T rich domains or intron splicing consensus sequences.

U. S. Patent 5,500,365 (specifically incorporated herein by reference) describes the preferred method for synthesizing plant genes to optimize the expression level of the protein for which the synthesized gene encodes. This method relates to the modification of the structural gene sequences of the exogenous transgene, to make them more "plant-like" and therefore more likely to be translated and expressed by the plant, monocot or dicot. However, the method as disclosed in U. S. Patent 5,689,052 provides for enhanced expression of transgenes, preferably in monocotyledonous plants, which is herein incorporated in its entirety by reference. Briefly, according to Brown et al., the frequency of rare and semi-rare monocotyledonous codons in a polynucleotide sequence encoding a desired protein are reduced and replaced with more preferred monocotyledonous codons. Enhanced accumulation of a desired polypeptide encoded by a modified polynucleotide sequence in a monocotyledonous plant is the result of increasing the frequency of preferred codons by analyzing the coding sequence in successive six nucleotide fragments and altering the sequence based on the frequency of appearance of the six-mers as to the frequency of appearance of the rarest 284, 484, and 664 six-mers in monocotyledenous plants. Furthermore, Brown et al. disclose the enhanced expression of a recombinant gene by applying the method for reducing the frequency of rare codons with methods for reducing the occurrence of polyadenylation signals and intron splice sites in the nucleotide sequence, removing self-complementary sequences in the nucleotide sequence and replacing such sequences with nonself-complementary nucleotides while maintaining a structural gene encoding the polypeptide, and reducing the frequency of occurrence of 5'-CG-3' di-nucleotide pairs in the nucleotide sequence. These steps are performed sequentially and have a cumulative effect resulting in a nucleotide sequence containing a preferential utilization of the more-preferred monocotyledonous codons for monocotyledonous plants for a majority of the amino acids present in the desired polypeptide.

Thus, the amount of a gene coding for a polypeptide of interest can be increased in plants by transforming those plants using transformation methods such as those disclosed herein. In

particular, chloroplast or plastid transformation can result in desired coding sequences being present in up to about 10.000 copies per cell in tissues containing these subcellular organelle structures (McBride et al., Bio/Technology 13:362-365, 1995).

DNA can also be introduced into plants by direct DNA transfer into pollen as described (Zhou *et al.*, 1983; Hess, 1987). Expression of polypeptide coding genes can be obtained by injection of the DNA into reproductive organs of a plant as described (Pena *et al.*, 1987). DNA can also be injected directly into the cells of immature embryos and introduced into cells by rehydration of desiccated embryos as described (Neuhaus *et al.*, 1987; Benbrook *et al.*, 1986).

After effecting delivery of exogenous DNA to recipient cells, the next step to obtain a transgenic plant generally concern identifying the transformed cells for further culturing and plant regeneration. As mentioned herein, in order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. In this case, one would then generally assay the potentially transformed cell population by exposing the cells to a selective agent or agents, or one would screen the cells for the desired marker gene trait.

An exemplary embodiment of methods for identifying transformed cells involves exposing the transformed cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells which have been transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing. One example of a preferred marker gene confers resistance to glyphosate. When this gene is used as a selectable marker, the putatively transformed cell culture is treated with glyphosate. Upon treatment, transgenic cells will be available for further culturing while sensitive, or non-transformed cells, will not. This method is described in detail in U. S. Patent 5,569,834, which is specifically incorporated herein by reference. Another example of a preferred selectable marker system is the neomycin phosphotransferase (*npt*II) resistance system by which resistance to the antibiotic kanamycin is conferred, as described in U. S. Patent 5,569,834 (specifically incorporated herein by reference). Again, after transformation with this system, transformed cells will be available for further culturing upon treatment with kanamycin, while non-transformed cells will not. Yet another preferred selectable marker system involves the use of a gene construct conferring resistance to

paromomycin. Use of this type of a selectable marker system is described in U. S. Patent 5,424,412 (specifically incorporated herein by reference).

Another preferred selectable marker system involves the use of the genes contemplated by this invention. In particular, a *phn*O gene or a substantially similar gene encoding an AMPA transacylase can be utilized as a selectable marker. Plant cells which have had a recombinant DNA molecule introduced into their genome can be selected from a population of cells which failed to incorporate a recombinant molecule by growing the cells in the presence of AMPA. One skilled in the art will recognize the particular advantages that this selectable marker system has over previous selectable marker systems. The selectable marker used in the recombinant DNA integrated into a plant genome reduces the amount of DNA targeted for integration because the selectable marker will also be used for improved herbicide tolerance or improved herbicide resistance in plants generated from transformed plant cells. This selectable marker also provides an additional marker system not known before, particularly in a field in which there are often only a limited number of selectable markers available.

Transplastonomic selection (selection of plastid or chloroplast transformation events) is simplified by taking advantage of the sensitivity of chloroplasts or plastids to spectinomycin, an inhibitor of plastid or chloroplast protein synthesis, but not of protein synthesis by the nuclear genome encoded cytoplasmic ribosomes. Spectinomycin prevents the accumulation of chloroplast proteins required for photosynthesis and so spectinomycin resistant transformed plant cells may be distinguished on the basis of their difference in color: the resistant, transformed cells are green, whereas the sensitive cells are white, due to inhibition of plastid-protein synthesis. Transformation of chloroplasts or plastids with a suitable bacterial *aad* gene, or with a gene encoding a spectinomycin resistant plastid or chloroplast functional ribosomal RNA provides a means for selection and maintenance of transplastonomic events (Maliga, Trends in Biotechnology **11**:101-106, 1993).

It is further contemplated that combinations of screenable and selectable markers will be useful for identification of transformed cells. In some cell or tissue types a selection agent, such as glyphosate or kanamycin, may either not provide enough killing activity to clearly recognize transformed cells or may cause substantial nonselective inhibition of transformants and nontransformants alike, thus causing the selection technique to not be effective. It is proposed that selection with a growth inhibiting compound, such as glyphosate at concentrations below

those that cause 100% inhibition followed by screening of growing tissue for expression of a screenable marker gene such as kanamycin would allow one to recover transformants from cell or tissue types that are not amenable to selection alone. It is proposed that combinations of selection and screening may enable one to identify transformants in a wider variety of cell and tissue types. The availability of the transacylases of the present invention may obviate the necessity for combination selection and screening by providing an additional selection means.

The development or regeneration of plants from either single plant protoplasts or various explants is well known in the art (Weissbach and Weissbach, 1988). This regeneration and growth process typically includes the steps of selection of transformed cells, culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil.

The development or regeneration of plants containing the foreign, exogenous gene that encodes a polypeptide of interest introduced by *Agrobacterium* from leaf explants can be achieved by methods well known in the art such as described (Horsch *et al.*, 1985). In this procedure, transformants are cultured in the presence of a selection agent and in a medium that induces the regeneration of shoots in the plant strain being transformed as described (Fraley *et al.*, 1983). In particular, U. S. Patent 5,349,124 (specification incorporated herein by reference) details the creation of genetically transformed lettuce cells and plants resulting therefrom which express hybrid crystal proteins conferring insecticidal activity against *Lepidopteran* larvae to such plants.

This procedure typically produces shoots within two to four months and those shoots are then transferred to an appropriate root-inducing medium containing the selective agent and an antibiotic to prevent bacterial growth. Shoots that rooted in the presence of the selective agent to form plantlets are then transplanted to soil or other media to allow the production of roots. These procedures vary depending upon the particular plant strain employed, such variations being well known in the art.

Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants, or pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important, preferably inbred lines. Conversely, pollen from plants of those important lines is used to pollinate regenerated plants. A transgenic plant of the present

invention containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

In one embodiment, a transgenic plant of this invention thus has an increased amount of a coding region encoding an AMPA transacylase polypeptide which may also be expressed along with a plastid targeting peptide. A preferred transgenic plant is an independent segregant and can transmit that gene and its activity to its progeny. A more preferred transgenic plant is homozygous for that gene, and transmits that gene to all of its offspring on sexual mating. Seed from a transgenic plant may be grown in the field or greenhouse, and resulting sexually mature transgenic plants are self-pollinated to generate true breeding plants. The progeny from these plants become true breeding lines that are evaluated for expression of the transacylase transgene as well as for improved herbicide tolerance, particularly when the transacylase transgene is co-expressed along with a gene encoding a GOX enzyme.

The genes and acyltransferases according to the subject invention include not only the full length sequences disclosed herein but also fragments of these sequences, or fusion proteins, which retain the characteristic improved herbicidal protective activity of the sequences specifically exemplified herein.

It should be apparent to a person of skill in this art that AMPA transacylase genes and peptides can be identified and obtained through several means. The specific genes, or portions thereof, may be obtained from a culture depository, or constructed synthetically, for example, by use of a gene machine. Variations of these genes may be readily constructed using standard techniques for making point mutations. Also, fragments of these genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as *Bal*31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes which code for active fragments may be obtained using a variety of other restriction enzymes. Proteases may be used to directly obtain active fragments of such transacylases.

Equivalent AMPA transacylases and/or genes encoding these transacylases can also be isolated from *E. coli* strains and/or DNA libraries using the teachings provided herein. For example, antibodies to the transacylases disclosed and claimed herein can be used to identify and isolate other transacylases from a mixture of proteins. Specifically, antibodies may be raised to the transacylases disclosed herein and used to specifically identify equivalent AMPA

transacylases by immunoprecipitation, column immuno-purification, enzyme linked immunoassay (ELISA), or Western blotting.

A further method for identifying the peptides and genes of the subject invention is through the use of oligonucleotide probes. These probes are nucleotide sequences having a detectable label. As is well known in the art, if the probe molecule and sequences in a target nucleic acid sample hybridize by forming a strong bond between the two molecules, it can be reasonably assumed that the probe and target sample contain essentially identical polynucleotide sequences. The probe's detectable label provides a means for determining in a known manner whether hybridization has occurred. Such a probe analysis provides a rapid method for identifying AMPA transacylase genes of the subject invention.

The nucleotide segments which are used as probes according to the invention can be synthesized by use of DNA synthesizers using standard procedures. In the use of the nucleotide segments as probes, the particular probe is labeled with any suitable label known to those skilled in the art, including radioactive and non-radioactive labels. Typical radioactive labels include ^{32}P, ^{125}I, ^{35}S, or the like. A probe labeled with a radioactive isotope can be constructed from a nucleotide sequence complementary to the DNA sample by a conventional nick translation reaction, using a DNase and DNA polymerase. The probe and sample can then be combined in a hybridization buffer solution and held at an appropriate temperature until annealing occurs. Thereafter, the membrane is washed free of extraneous materials, leaving the sample and bound probe molecules typically detected and quantified by autoradiography and/or liquid scintillation counting.

Non-radioactive labels include, for example, ligands such as biotin or thyroxin, as well as enzymes such as hydrolyses or peroxidases, or the various chemiluminescers such as luciferin, or fluorescent compounds like fluorescein, rhodamine, Texas Red, and derivatives and the like. The probe may also be labeled at both ends with different types of labels for ease of separation, as, for example, by using an isotopic label at the end mentioned above and a biotin label at the other end, or with different fluorescent emitters which have overlapping absorption and emission spectra.

Duplex formation and stability depend on substantial complementary between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probes of the subject invention include mutations (both single and multiple),

deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, by methods currently known to an ordinarily skilled artisan, and perhaps by other methods which may become known in the future.

The potential variations in the probes listed is due, in part, to the redundancy of the genetic code. Because of the redundancy of the genetic code, more than one coding nucleotide triplet (codon) can be used for most of the amino acids used to make proteins. Therefore different nucleotide sequences can code for a particular amino acid. Thus, the amino acid sequence of the *E. coli* AMPA transacylase and peptide, and the plastid targeting peptides and the polynucleotides which code for them, can be prepared by equivalent nucleotide sequences encoding the same amino acid sequence of the protein or peptide. Accordingly, the subject invention includes such equivalent nucleotide sequences. Also, inverse or complement sequences are an aspect of the subject invention and can be readily used by a person skilled in this art. In addition it has been shown that proteins of identified structure and function may be constructed by changing the amino acid sequence if such changes do not alter the protein secondary structure (Kaiser and Kezdy, 1984). Thus, the subject invention includes mutants of the amino acid sequence depicted herein which do not alter the protein secondary structure, or if the structure is altered, the biological activity is substantially retained. Further, the invention also includes mutants of organisms hosting all or part of a gene encoding an AMPA acyltransferase and/or gene encoding a plastid targeting peptide, as discussed in the present invention. Such mutants can be made by techniques well known to persons skilled in the art. For example, UV irradiation can be used to prepare mutants of host organisms. Likewise, such mutants may include asporogenous host cells which also can be prepared by procedures well known in the art.

Site-specific or site-directed mutagenesis is a technique useful in the preparation of individual, novel and unique useful peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of structural genes encoding such peptides. The technique further provides a ready ability to prepare and test sequence variants by altering the coding sequence of a gene, for example, by introducing one or more nucleotide sequence changes into the DNA for the purpose of creating a new or useful restriction endonuclease

cleavage recognition sequence or for the purpose of altering the coding sequence so that a gene's codons and percent G/C represent those more commonly used by a particular genus or species. Site-specific mutagenesis allows the production of deletion, insertion, or replacement mutations through the use of specific mutagenesis oligonucleotide sequences comprising the DNA sequence of the desired mutation. Mutagenesis oligonucleotides typically provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the desired mutation target site. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues overlapping either side of the desired mutation target site.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, and often contain a filamentous phage origin of replication which, in the presence of a helper phage, allows synthesis of single stranded DNA from the plasmid vector.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double stranded vector which includes within its sequence a mutation target site. A mutagenesis oligonucleotide primer bearing the desired mutant sequence is prepared, generally synthetically. The mutagenesis primer is then annealed with the single-stranded vector at the mutation target site, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors containing the mutation represented by the mutagenesis primer sequence.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors

encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Such procedures may favorably change the protein's biochemical and biophysical characteristics or its mode of action. These include, but are not limited to: 1) improved AMPA transacylase formation, 2) improved protein stability or reduced protease degradation, 3) improved substrate recognition and binding, 4) improved enzyme kinetics, and 5) improved N-acyl-AMPA formation due to any or all of the reasons stated above.

Modification and changes may be made in the structure of the peptides of the present invention and DNA segments which encode them and still obtain a functional molecule that encodes a protein or peptide with desirable characteristics. The biologically functional equivalent peptides, polypeptides, and proteins contemplated herein should possess at least from about 40% to about 65% sequence similarity, preferably from about 66% to about 75% sequence similarity, more preferably from about 76% to about 85% similarity, and most preferably from about 86% to about 90% or greater sequence similarity to the sequence of, or corresponding moiety within, the AMPA acyltransferase amino acid sequences disclosed herein.

The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. In particular embodiments of the invention, mutated AMPA transacylase proteins are contemplated to be useful for improving or enhancing the *in planta* expression of the protein, and consequently increasing or improving the AMPA transacylase activity and/or expression of the recombinant transgene in a plant cell. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to the codons given in Table 1, in dicotyledonous, and more particularly in monocotyledonous plants.

Table 1

Amino Acid			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventor that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by

reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (–0.4); threonine (–0.7); serine (–0.8); tryptophan (–0.9); tyrosine (–1.3); proline (–1.6); histidine (–3.2); glutamate (–3.5); glutamine (–3.5); aspartate (–3.5); asparagine (–3.5); lysine (–3.9); and arginine (–4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (–0.4); proline (–0.5 ± 1); alanine (–0.5); histidine (–0.5); cysteine (–1.0); methionine (–1.3); valine (–1.5); leucine (–1.8); isoleucine (–1.8); tyrosine (–2.3); phenylalanine (–2.5); tryptophan (–3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity,

hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

Polynucleotides encoding heterologous proteins are known by those skilled in the art, to often be poorly expressed when incorporated into the nuclear DNA of transgenic plants (reviewed by Diehn *et al.*, 1996). Preferably, a nucleotide sequence encoding a heterologous protein of interest is designed essentially as described in U. S. Patent 5,500,365 and 5,689,052 (each specifically incorporated herein by reference). Examples of nucleotide sequences useful for expression include but are not limited to, SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:11, and SEQ ID NO:19.

Substitutes for an amino acid within the fundamental polypeptide sequence can be selected from other members of the class to which the naturally occurring amino acid belongs. Amino acids can be divided into the following four groups: (1) acidic amino acids; (2) basic amino acids; (3) neutral polar amino acids; and (4) neutral non-polar amino acids. Representative amino acids within these various groups include, but are not limited to: (1) acidic (negatively charged) amino acids such as aspartic acid and glutamic acid; (2) basic (positively charged) amino acids such as arginine, histidine, and lysine; (3) neutral polar amino acids such as glycine, serine, threonine, cyteine, cystine, tyrosine, asparagine, and glutamine; (4) neutral nonpolar (hydrophobic) amino acids such as alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine.

Conservative amino acid changes within a fundamental polypeptide sequence can be made by substituting one amino acid within one of these groups with another amino acid within the same group. The encoding nucleotide sequence (gene, plasmid DNA, cDNA, or synthetic DNA) will thus have corresponding base substitutions, permitting it to encode biologically functional equivalent forms of an AMPA transacylase.

The following examples describe preferred embodiments of the invention. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art of endeavor from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the

examples. In the examples all percentages are given on a weight basis unless otherwise indicated.

EXAMPLES

Example 1

This example illustrates the growth inhibitory effects of N-aminomethyl phosphonic acid (AMPA) on plant callus tissue, and the lack of inhibition of N-acetyl-aminomethyl phosphonic acid on plant callus tissue in *in vitro* culture conditions.

Certain recombinant plant species which express a bacterial GOX gene, and which were also exposed to glyphosate, can exhibit phytotoxic effects manifested through such symptoms as chlorosis, flower abscission, and reduced fertility. The basis for these symptoms had not previously been determined. Previous studies had indicated that plants expressing GOX metabolized glyphosate to AMPA and glyoxylate (U.S. Patent No. 5,463,175). Glyoxylate is readily metabolized by plants, however AMPA persists in plant tissues and may be the cause of phytotoxic effects such as chlorosis, stunting, or other undesireable effects. It had previously been shown that *Achromobacter* species LBAA was able to enzymatically modify AMPA to N-acetyl AMPA (U.S. Patent No. 5,463,175). The *Achromobacter* data, coupled with the plant phytotoxicity data, indicated that N-acylation of AMPA *in planta* may provide effective relief from chlorosis and other undesireable effects. Thus, tobacco callus tissue was exposed to AMPA and to N-acetyl AMPA in order to determine if either of these compounds exhibited cytotoxic effects similar to those observed in plants expressing GOX and exposed to glyphosate.

Tobacco callus was generated from leaf pieces of wild type *Nicotiana tabacum* cv. "Samsun" tobacco on MS104 plates (MS salts 4.3 g/l, sucrose 30 g/l, B5 vitamins 500X 2 ml/l, NAA 0.1 mg/l, and Bacto Agar 1.0 mg/l). Callus tissue was applied to plates with or without AMPA and with or without N-acetyl AMPA. Plates contained AMPA or N-acetyl AMPA at concentrations of 0.1 mM or 0.4 mM. Plates were incubated for up to three weeks and monitored periodically.

Callus tissue on control plates containing no AMPA or N-acetyl AMPA grew at normal rates, regenerating roots and shoots as expected. Callus tissue in the presence of AMPA was severely inhibited. No growth was observed, showing the phytotoxic effect of AMPA at these concentrations. Callus tissue on plates containing N-acetyl AMPA was not inhibited, and formed roots and shoots similar to control callus tissue growth. This result indicated that

AMPA, as a byproduct of GOX mediated metabolism of glyphosate, could be responsible for the observed phototoxicity in plants. This result also indicated the possibility of an improved method for selecting plants from genetically transformed callus tissue, as well as a possible method for enhancing glyphosate herbicide resistance.

Example 2

This example illustrates that degradation of glyphosate by GOX enzyme hydrolysis in the bacterium *Achromobacter* sp. strain LBAA results in the production of AMPA and N-acetyl AMPA.

It has been previously shown that GOX mediated glyphosate degradation produced glyoxylate and AMPA (Barry et al., US 5,463,175). *Achromobacter* sp. strain LBAA was also shown to produce AMPA and glyoxylate as a result of glyphosate degradation. The glyphosate degradation pathway was characterized in resting cells of glyphosate-grown *Achromobacter* sp. strain LBAA according to the following procedure. Cells from a 100 ml culture of LBAA, grown in DF3S medium containing glucose, gluconate and citrate as carbon sources and with thiamine and Yeast Extract (0.01%) to supply trace requirements and with glyphosate at 0.2 mM as a phosphorous source, were harvested at a cell density of 200 Klett units, washed twice with 20 ml of DF3S medium and the equivalent of 20 ml of cells were resuspended in 100 l of the same medium containing [^{14}C]glyphosate (2.5 ml of 52 mCi/mmol, Amersham; CFA.745). The cell mix was incubated at 30°C with shaking and 20ml samples were withdrawn at various intervals. The samples were centrifuged to separate the cells from the broth supernatant. Both the supernatant and cell pellets were analyzed by HPLC.

Samples prepared in this way were analyzed by strong anion exchange (SAX) HPLC with radioisotope label detection to determine their levels of [^{14}C]-AMPA and N-acetyl-[^{14}C]-AMPA. Samples were injected using a Waters WISP autoinjector. Chromatographic profiles and quantitative data were collected using MACS2, Monsanto's automated chromatography data collection system. A Spherisorb S5 SAX, 250 mm X 10mm column, or an Alltech 5 micron, 250mmX10mm SAX column was used for the analyses. Solvents used were designated as solution A and solution B. Solution A contained 0.005M KH_2PO_4 adjusted to pH 2.0 with H_3PO_4 in 4% methanol. Solution B contained 0.10 M KH_2PO_4 adjusted to pH 2.0 with H_3PO_4 in 4% methanol. Each sample run time consisted of a step gradient program with an eluent flow rate of 3 ml per minute and a scintillation fluid (tradename ATOMFLOW, No. NEN-995

obtained from Packard Instruments) flow rate of 9 ml per minute. The HPLC solvent profile for distinguishing [^{14}C]-AMPA from N-acetyl-[^{14}C]-AMPA in each sample analyzed was represented by 100% solvent A at times zero through 5 minutes, then solvent B at 100% at time 5 minutes through 15 minutes, then 100% solvent A through 20 minutes at which time the column is prepared to receive another sample.

Cell pellets were first resuspended in DF3S medium made acidic by addition of 0.65N HCl, boiled for 5 minutes, then centrifuged briefly to provide a solution phase for HPLC analysis. Supernatants were treated similarly prior to HPLC analysis. An acidified glyphosate control was also subjected to HPLC analysis, and the glyphosate retention time (RT) was determined to be 10.8 minutes. The amount of radioactivity in the glyphosate peak remaining in the supernatant after two hours incubation had decreased to about 33% of the initial levels, indicating that the glyphosate was extensively metabolized. About 3% of the glyphosate was found to be within the cell. Material co-eluting with the methylamine standard with an RT of 6 minutes accounted for about 5% of the initial amount of radioactivity in the supernatant and for about 1.5% of the initial amount of radioactivity identified in the cell contents.

The GOX mediated glyphosate degradation pathway was elucidated further in a subsequent experiment where the metabolism of [^{14}C]AMPA was compared to that of [^{14}C]glyphosate as indicated above in resting cells harvested at 165 Klett units and resuspended at the equivalent of 15 ml cells per 100 ml DF3S medium. The samples were analyzed by HPLC and consisted of whole cultures acidified and treated as described above. Cultures exposed to [^{14}C]glyphosate for two hours were found to have 25% of the label in the methylamine/N-acetyl-methylamine peak with a retention time of 14.7 minutes, 12.5% as AMPA with a retention time of 6 minutes, 30% in a peak with a retention time of 13.2 minutes, and 30% as glyphosate with a retention time of 10.8 minutes. Analysis of cultures exposed to [^{14}C]-AMPA for two hours indicated that 15% of the label was found as N-acetyl-methylamine / methylamine, 59% as AMPA, and 18% in the 13.2 minute peak. The material eluting at 13.2 minutes was identified as N-acetyl-AMPA by negative ion electrospray mass spectrometry. The result showed strong ions at m/e 152 and m/e 154, as expected for this compound, which has a molecular weight of 153 Daltons. The m/e 154 ion was due to the isotopic ^{14}C atom. N-acetyl-methyl-[^{14}C]-AMPA arises from N-methyl-[^{14}C]-AMPA, which is a known impurity in preparations of [^{14}C]-AMPA.

These data indicated that the glyphosate degradation pathway in *Achromobacter* strain LBAA proceeds from hydrolysis of glyphosate to AMPA, which is then converted to the products methylamine presumably through a dephosphorylation step, and N-acetyl-AMPA presumably through some previously unknown transacylation step. A small amount of N-acetyl-AMPA is then converted to N-acetyl-methylamine. A similar acylation step has been inferred from the products identified in *E. coli* when aminomethylphosphonates are utilized as sole sources of phosphate (Avila et al., 1987).

Example 3

This example illustrates the identification of an AMPA acyltransferase activity in *E. coli*.

Avila et al. (1987) identified dephosphorylated biodegradation products from the metabolism of a variety of aminophosphonate substrates used as sole phosphate sources *in vivo* in *E. coli* while studying C-P bond scission. Their studies indicated that AMPA was not a substrate for acylation in *E. coli* K-12. In addition, Avila et al. were interested in the effect of N-linked chemical substitutions on C-P bond scission of phosphonates in *E. coli*, and identified N-acetylated products derived from the metabolism of some aminophosphonates. Avila et al. also demonstrated that 'wild type' *E. coli* K12 strains, unlike wild type *E. coli* B strains, are unable to use phosphonates as a source of phosphate. Thus, in consideration of the phytotoxic effects of AMPA on callus tissue as shown in Example 1 and the generation of AMPA from GOX mediated glyphosate degradation as shown in Example 2, the *E. coli* data in Avila et al. indicated that there may be an enzyme or pathway present in some bacterial species which is capable of converting aminomethylphosphonate (AMPA) to N-acetyl-AMPA. An enzyme or pathway with those characteristics would, if expressed in plants, confer a significant advantage to plants expressing GOX when treated with glyphosate.

To test this, an *E. coli* K-12 strain adapted for growth on AMPA was grown on low phosphate containing medium in order to obtain cell lysates to be assayed for the presence of an enzyme capable of AMPA N-acylation. The *phn* (*mpu*) operon is cryptic in *E. coli* K-12 due to an 8 base pair insertion which causes a frameshift mutation in the *phn*E gene. The frameshift inactivates PhnE and creates a polar effect on translation of other genes downstream of *phn*E within the operon, resulting in the inability of such mutants to use phosphonates as phosphate sources (Makino et al., J. Bacteriol. **173**:2665-2672, 1991). Selection of a spontaneously derived mutation restores the function of the *phn* operon (*phn*+ or *mpu*+). Thus, K-12 strains adapted

for growth on AMPA, methyl-phosphonate, or ethyl-phosphonate contain such effective spontaneously derived mutations.

Briefly, an aliquot of a fresh L-broth culture of *E. coli* K-12 strain JM101 (*mpu-*) was plated onto MOPS (Neidhardt et al., 1974) complete agar medium containing amino acids at 25mg/ml, vitamin B1 [thiamine] at 10 mg/ml, 0.2% glucose, and 1.5% DIFCO "Purified" agar along with aminomethylphosphonate (AMPA; 0.2 mM; Sigma Chemical Co., St. Louis, MO) as the sole phosphate source, and incubated at 37°C for three days. Colonies arising on this media were picked and streaked onto MOPS complete agar containing either AMPA or methylphosphonate (Alfa) as the sole phosphate source. One colony, designated *E. coli* JM101 *mpu+*, was chosen from those that grew equally and uniformly on both phosphonate containing media, and was further designated as *E. coli* strain GB993.

The *phn* operon is induced when *E. coli* is grown in media lacking or limited in a phosphate source. Therefore, *E. coli* GB993 was compared to the parental JM101 strain when grown in MOPS minimal media. GB993 and its *mpu-* parent strain, JM101, were grown under identical conditions, varying only the amount of phosphate available or supplemented with AMPA. 50 ml cultures were grown in duplicate in 250 ml sidearm-Erlenmeyer flasks with continuous shaking at 37°C in MOPS medium (5 mls of 10X MOPS salts, 0.5 ml 1 mg/ml thiamin, 0.5 ml 20% glucose, to 50 mls with dH_20) containing 0.1 or 5 mM phosphate, or 0.1 mM phosphate supplemented with approximately 0.2 mM AMPA, pH 7.0. The cultures were generally grown to about 220 Klett units and the cells were pelleted by centrifugation, resuspended in 1.5 mls of 10 mM Tris/1 mM DTT, and lysed with two passes through a French press at 1,000 psi. Lysates were centrifuged to remove debris and the supernatant passed through a G-50 column equilibrated with 50 mM Tris pH 7.0. Table 2 shows the results of cell cultures grown in this manner.

Table 2.

Effects of Phosphate Substrate on Cell Growth

	Strain					
	JM101	JM101	JM101	GB993	GB993	GB993
	0.1 mM Phosphate	5 mM Phosphate	0.2 mM AMPA	0.1mM Phosphate	5 mM Phosphate	0.2 mM AMPA
Growth Period (hrs)	48	29	54	48	29	54
Harvest Density (Klett Units)	155	240	-	140	244	185

- indicates no measurable growth

An HPLC assay was used to determine the presence or absence of any AMPA acyltransferase activity in the media and cell lysates. The assay monitors the conversion of [^{14}C]AMPA to N-acetyl-[^{14}C] AMPA. Generally, 100 µl of a 2X assay solution consisting of 16.5 mg acetyl-CoA, 250 µl of 2M Tris, pH 7.5, 4.5 mls dH_20 and [^{14}C]AMPA (30mM) was mixed with 25-75 µl of lysate and 1 µl each of 0.5 M $MgCl_2$ and $MnCl_2$, and brought to 200 µl with dH_20. The assay was incubated for 30 minutes at 37°C, and quenched with 200 µl 90-100 mM NaOAc (sodium acetate) pH 4.4 in ethanol and then analyzed immediately by HPLC as described above, or stored at -20°C. Only GB993 lysate samples derived from cultures grown in the presence of AMPA or 0.1 mM phosphate supplemented media demonstrated appreciable AMPA acyltransferase activity. This result indicated that a gene encoding an acyltransferase enzyme capable of AMPA N-acylation was present in GB993 and was regulated for expression when grown under low phosphate conditions. Thus, the coding sequence for the enzymatic activity appears to be part of the *pho* regulon and may reside in the *phn* operon.

Example 4

This example illustrates the identification of an *E. coli phn* operon gene encoding an enzyme capable of AMPA acylation.

Example 3 indicated that the AMPA acyltransferase activity observed in lysates of *E. coli* may be encoded by a gene in the *phn* operon. The entire *phn* operon in *E. coli* B and in *E. coli* K-12 has previously been cloned and sequenced B (Wanner et al., Chen et al.). The *E. coli* K-12 *phn* operon DNA sequence has been shown to be identical to the published DNA sequence of the *phn* operon from *E. coli* B with the exception of an eight base pair insertion in the *phn*E gene (Wanner et al). Clones containing various amounts of the *phn* operon genes from either bacterial genetic background are readily available (Wanner et al., Chen et al., Dr. J.W. Frost at Purdue University). Plasmids containing differing amounts of the JM101 *phn* operon DNA were used to transform JM101(*mpu-*) in order to test for a plasmid localized *phn* gene that, when expressed, confers upon JM101 the ability to utilize AMPA as a sole phosphate source.

A plasmid obtained from J. Frost (Dr. J.W. Frost, Department of Chemistry, Purdue University, West Lafayette, Indiana 47907), designated herein as pF, contains an *E. coli* K-12 8 kb *Eco*RI fragment which encodes the *phn* operon genes ph*n*G through *phn*Q. A single *Nco*I site is present at the 5' end of the *phn*G coding region. Plasmid pF was digested with *Eco*RI and *Nco*I, releasing a 2 kb *Nco*I-*Eco*RI fragment containing the genes *phn*G through *phn*I, and a

second *Nco*I-*Eco*RI fragment about 6 kb in length containing the genes *phn*J through *phn*Q. Each fragment was gel purified and ligated into a cloning and expression vector in an orientation which would allow for expression of the *phn* operon genes present within each of the *Nco*I-*Eco*RI fragments from a plasmid borne inducible promoter. The 2 kb fragment was inserted into the *Nco*I - *Eco*RI sites within the vector pMON7258, a positive selection cloning vector identical to pUC118 with the exception of polylinker domain (Viera et al., Methods Enzymol. **153**:3, 1987), the resulting plasmid being designated as p58-1. The orientation of the 2 kb fragment in p58-1 allows for the expression of the *phn*G-*phn*I genes from the *lac* promoter within the vector. The 6 kb *Eco*RI-*Nco*I fragment was inserted into the *Nco*I and *Eco*RI sites in a similar positive selection vector, pMON7259, producing the plasmid designated as pMON17195. pMON7259 is identical to pUC119 except for the polylinker domain, which contains a multiple cloning site opposite in orientation to that within pMON7258, and which also allows for expression of the *phn*J-*phn*Q genes from a *lac* promoter. p58-1 and pMON7259 were transformed into *E. coli* K12 (*mpu*-) strain JM101, and maintained with ampicillin antibiotic resistance selection. pMON7259 and pF were also transformed into JM101 as negative and positive controls, respectively.

Cultures of each transformant were grown overnight in M9 liquid broth media supplemented with 2% casamino acids, thiamine, and 0.2% glucose with shaking at 37°C, and then diluted 1:50 into 50 ml of fresh pre-warmed media of the same composition in a 250 ml side-armed Erlenmeyer flask. Cultures were incubated with shaking at 37°C until reaching a cell density of about 80-100 Klett Units as measured on a Klett-Summerson spectrophotometer through a #2 green filter. Expression from the plasmid *lac* promoter was induced by the addition of 100 microliters of 500 mM IPTG so that the final IPTG concentration was about 1 mM. The induction phase growth period was allowed to progress for two hours. Table 3 shows the cell density profile of each culture from 1:50 dilution through the two hour induction period.

Table 3.

Induction Profile of JM101 Cultures Harboring Various *phn* Plasmids

Culture/ Plasmid	IPTG	I_0	I_1	I_2
pMON7259	+	13	75	222
p58-1	+	15	70	212
pMON17195	+	15	90	220
pF	+	17	97	290
pF	-	15	-	260

I_0 indicates the cell culture density at the 1:50 dilution time point; I_1 indicates the cell culture density at the time of IPTG addition; and I_2 indicates the cell culture density at the time of harvest.

The cells in each culture were harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C in a Beckman J2 centrifuge. The cell pellet was washed one time in ice cold 154 mM NaCl solution, and then resuspended in 1.5 ml extraction buffer (50 mM Tris-HCl pH 7.5, 1 mM DTT, 50 mM Tris-HCl pH 7.5). Cell suspensions were ruptured with two passes through a French Press at 1000 psi. The resulting lysate was centrifuged for 15 minutes at 14,000 rpm at 4°C in an EPPENDORF™ model 5402 microcentrifuge in order to remove debris. Each cleared lysate was transferred to a fresh pre-chilled tube and the volume of the extract was adjusted to 2.5 ml with 50 mM Tris-HCl pH 7.5. A PD10 column was equilibrated with 25 ml 50 mM Tris-HCl, pH 7.5 and then each sample was applied to the desalting column. Each eluted sample was adjusted to 3.5 ml with 50 mM Tris-HCl, pH 7.5. Each sample was distributed to assay tubes and mixed with reagents in order to assay for the presence of AMPA acyltransferase activity as shown in Table 4.

Table 4.

Assay Conditions for Bacterial Lysates Expressing *phn* Genes

Sample	IPTG	Extract Volume*	50 mM Tris Volume*	2X Assay Mix Volume*	Total Volume*
pMON7259	+	25	75	100	200
pMON7259	+	100	0	100	200
p58-1	+	25	75	100	200
p58-1	+	100	0	100	200
pMON17195	+	25	75	100	200
pMON17195	+	100	0	100	200
pF	+	25	75	100	200
pF	+	100	0	100	200
pF	-	25	75	100	200
pF	-	100	0	100	200
-	na	0	100	100	200

*all volumes are in microliters

Composition of mixtures of each sample, designated by plasmid content, as prepared for AMPA acyltransferase assay.

Each mixture was incubated at 37°C for 30 minutes, and quenched with an equal volume (200 microliters) of 90-100 mM NaOAc (sodium acetate), pH 4.4 in ethanol and if not analyzed immediately by HPLC as described above, then stored overnight at -20°C. Unused portions of each lysate were stored either at 4°C, or mixed with glycerol to 10% by volume, and stored at -20°C.

Samples of each lysate subjected to the AMPA transacylase assay were analyzed by HPLC for the presence of [^{14}C]AMPA and acylated [^{14}C]AMPA, as described above. The results are shown in Table 5.

Table 5.

HPLC Analysis of Bacterial Lysate Conversion of AMPA to Acetyl-AMPA

Sample	%Acetyl AMPA	%AMPA
pMON7259	no data	no data
pMON7259	8	92
p58-1	5	95
p58-1	13	87
pMON17195	100	0
pMON17195	100	0
pF	61	39
pF	97	3
pF	52	48
pF	90	10
-	-	100

Results of HPLC analysis of each sample, indicating the relative amount of [^{14}C] AMPA or acetyl-[^{14}C]AMPA as a percentage of the total amount of [^{14}C] in both peaks combined.

This data indicated that the plasmid containing the 6 kb *Nco*I-*Eco*RI fragment isolated from pF in pMON17195 contained one or more genes which, upon IPTG induction of the *lac* promoter in an *mpu-* strain of *E. coli*, elicited the production of an acyltransferase activity capable of converting all of the [^{14}C]AMPA available in the assay mix to acetyl-[^{14}C]AMPA. The gene or genes required for AMPA N-acylation were further defined by restriction deletion analysis.

Plasmids containing various segments of the *phn* operon from either *E. coli* B or *E. coli* K-12 were constructed to further delineate the nature of the *phn* operon gene or genes involved

in conferring AMPA acyltransferase activity when expressed in an *mpu- E. coli* JM101. pMON7333 contains the pMON17195 equivalent *E. coli* DNA insertion, but in pUC119, and is a single *E. coli* B strain *Hind*III fragment containing the wild type *phn* operon genes *phn*G through *phn*Q. pMON15020 was constructed by cloning a 5,713 base pair *Nco*I to *Eco*RI *E. coli* B DNA fragment from pMON7333 into pMON7259, and contains the genes *phn*J through *phn*Q. pMON15022 was constructed by inserting a 1,686 base pair *Eco*RI to *Sal*I fragment from pMON17195 into the positive selection cloning and expression vector pBlueScriptSP (Invitrogen), which contains the *E. coli* K-12 genes *phn*O, P and Q. pMON15023 was constructed by deleting an 1,820 base pair *Sal*I fragment from pMON17195, leaving behind the *E. coli* K-12 *phn* operon genes *phn*J and *phn*K, the 5'end of *phn*L, and all of *phn*O, P and Q.

The plasmids pMON17195, pMON15020, pMON15022, pMON15023, and pMON7259 were transformed into the *mpu- E. coli* K-12 strain JM101 and were maintained by ampicillin antibiotic selection. Overnight cultures of each of these transformants were grown with antibiotic selection and were diluted 1:50 into fresh M9 media as described above, and incubated at 37°C with shaking in 250 ml sidearm-Erlenmeyer flasks to a cell density of about 100 Klett units. Each culture was induced with IPTG as in example 3, and incubated for two additional hours with shaking. The cells were harvested by centrifugation in a Beckman J2 centrifuge at 4,000 RPM for 10 minutes at 4°C. Cell pellets were washed once with 50 ml of 154 mM NaCl, and stored at -20°C.

Cell pellets were resuspended in 1.5 ml Extraction Buffer as in example 3 and ruptured by two passes through a French Press at 1000 psi. The ruptured cell suspensions were centrifuged in an Eppindorf microcentrifuge Model 5402 for 15 minutes at 14,000 rpm and at 4°C. The cleared lysates were decanted into new tubes pre-chilled on ice, and the total volume was adjusted to 2.5 ml with addition of Extraction Buffer. These samples were desalted over a PD10 column pre-equilibrated with 25 ml of 50 mM Tris-HCl, pH 7.5, and eluted with 3.5 ml of 50 mM Tris HCl pH 7.5. Samples were then subjected to an AMPA acylation assay as described above, incubated for 30 minutes at 37°C, and quenched with 200 microliters of 90.9 mM NaOAc pH 4.4. The volumes of each sample used in the assay are noted in Table 6. All volumes represent microliters of each solution used.

Table 6.

Assay Conditions for Bacterial Lysates Expressing *phn* Genes from Plasmids

Plasmid	Extract	50 mM Tris	2X Assay Mix	Total Volume
-	-	100	100	200
pMON 17195	25	75	100	200
pMON 17195	100	-	100	200
pMON 15020	75	75	100	200
pMON 15020	100	-	100	200
pMON 15022	75	75	100	200
pMON 15022	100	-	100	200
pMON 15023	75	75	100	200
pMON 15023	100	-	100	200
pMON 7259	75	75	100	200
pMON 7259	100	-	100	200

Composition of mixtures of each sample, designated by plasmid content, as prepared for AMPA acyltransferase assay

Quenched samples were subjected to HPLC analysis as described above. Table 7 illustrates the results of HPLC analysis of each sample, indicating the relative amount of [^{14}C] AMPA or acetyl-[^{14}C]AMPA as a percentage of the total amount of [^{14}C] in both peaks combined.

Table 7.

HPLC Analysis of Bacterial Lysate [^{14}C]-AMPA Conversion to Acetyl-[^{14}C]-AMPA

Sample	Extract Volume	%[^{14}C]-AMPA	%Acetyl-[^{14}C]-AMPA	Total % [^{14}C]
-	-	100	-	100
pMON17195	25	66	34	100
pMON17195	100	26	74	100
pMON15020	75	-	100	100
pMON15020	100	-	100	100
pMON15022	75	-	100	100
pMON15022	100	-	100	100
pMON15023	75	-	100	100
pMON15023	100	-	100	100
pMON 7259	75	87	13	100
pMON 7259	100	72	28	100

HPLC analysis of each sample, indicating the relative amount of [^{14}C] AMPA or acetyl-[^{14}C]AMPA as a percentage of .he total amount of [^{14}C] in both peaks combined

The data in Table 7 indicates that AMPA acylation activity is derived from the *phn* operon open reading frames consisting of *phn*O, *phn*P, and *phn*Q, which are the only *phn* genes present in pMON15022. Other plasmids conferring AMPA acylation activity upon induction also contained at least the *phn*O, P, and Q genes, providing strong evidence that the observed activity was the result of one or more of these gene products. Therefore, additional plasmids were constructed based on the *phn*O, P, and Q gene sequences in order to determine which gene or genes were required for the acylation function.

Bacterial acylase, transacylase, and acyltransferase genes have been known in the literature for some time. Most are small 15-25 K Da proteins. Therefore, on the basis of size comparison, only the *phn*O and *phn*Q gene products would fall into this category. However, based on similarity comparisons with other proteins in the GENBANK, SWISSPROT, and EMBL databases, the predicted *phn*O gene product appeared to most closely resemble other proteins having acylase activity. For example, the *E. coli* PhnO protein aligned well with a gentamicin acetyltransferase-3-I described in Wohlleben et al. (Mol. Gen. Genet. 217:202-208, 1989). pMON15020 containing the *E. coli* B *phn* operon genes p*hn*J through *phn*P on a single 6.0 kb *Nco*I-*Eco*RI fragment was digested with *Sal*I *and Eco*RI to release a 2.0 kb fragment containing the *phn*O, P and Q genes. This 2 kb fragment was excised and purified from a 0.7% TAE Agarose gel , treated with T4 DNA polymerase to excise the 3' overhanging ends, then with Klenow and deoxynucleotide triphosphates (dXTP's) to provide blunt ends, and then ligated into the *Eco*RV site of pBlueScriptSP to produce plasmid pMON15024. pMON15024 was digested with *Nde*I and *Eco*RI, deleting a 1200 base pair fragment containing most of the *phn*P and all of the *phn*Q coding sequences. The remaining pMON15024 plasmid fragment still containing the *phn*O gene was treated with Klenow fragment DNA polymerase in the presence of dideoxynucleotides according to the manufacturer's instructions in order to fill in the 3' ends exposed by restriction enzyme digestion, then ligated together to produce the plasmid pMON15027. pMON15027 contains only the *phn*O gene flanked 3' by a small portion of *phn*P. The 1200 base pair *Nde*I to *Eco*RI fragment obtained from pMON15024 was cloned into pMON2123 to produce pMON15026, which contains the 3' two thirds of the *phn*P gene flanked 3' by *phn*Q. Plasmids pMON15024, 15026, and 15027 were introduced into *mpu-* JM101, and cell lysates of transformants were analyzed as above after growth and induction for the presence of AMPA acyltransferase activity. Only pMON15024 and pMON15027 exhibited

acyltransferase activity, indicating that the *phn*O gene product was responsible for AMPA acylation.

A DNA fragment containing only the *phn*O gene with convenient flanking restriction endonuclease sites for use in further cloning manipulations was produced using thermal cycling methods. Synthetic oligonucleotide primers were synthesized by Midland Certified Reagents, Co. (Midland Texas) based on the published *phn*O gene and flanking sequence in order to amplify the *phn*O gene (Chen et al., J. Biol. Chem. **256**: 4461-4471, 1990). The sequence AAACACCATGGCTGCTTGTG (SEQ ID NO: 5), designated AATPCR6, represents a synthetic oligonucleotide which is homologous to the template strand of the *phn*O gene. The 5' adenosine residue of SEQ ID NO: 5 corresponds to base pair 13,955 of the published *phn* operon sequence, immediately 5' of the *phn*O ATG initiation codon at position 13,962-13,964 (Chen et al., J. Biol. Chem. **256**: 4461-4471, 1990). SEQ ID NO: 5 incorporates a single base pair mismatch from the published *phn*O sequence at position 13,965 represented by a C to G inversion, which generates an alanine codon in place of a proline codon at position 2 and also creates a unique *Nco*I restriction site spanning the ATG initiation codon. The sequence GTGACGAATTCGAGCTCATTACAGCGCCTTGGTGA (SEQ ID NO: 6), designated AATPCR7, represents a synthetic oligonucleotide which is homologous to the coding strand of the *phn*O gene. The 3' adenosine residue of SEQ ID NO: 6 corresponds to base pair 14,380 of the published *phn* operon (Chen et al., J. Biol. Chem. **256**: 4461-4471, 1990). The thymidine at position number nineteen of SEQ ID NO: 6 corresponds to the adenosine at position 14,396 of the published *phnO* sequence (Chen et al.). A portion of SEQ ID NO: 6 overlaps the native *phn*O termination codon, introduces a second in frame termination codon immediately 3' of and adjacent to the native termination codon, and also introduces unique *Eco*RI and *Sac*I restriction sites 3' of these termination codons.

pMON15024 was used as a template for amplification of the *phn*O gene in a standard thermal amplification reaction. Briefly, a 100 microliter reaction sample was prepared which contained 0.1 ng template DNA, reaction buffer, 200 pM each primer, 200 mM dNTP, 1.25 U Taq DNA polymerase and was overlayed with mineral oil. This reaction sample was subjected to thirty five cycles at 94°C for one minute, 50°C for two minutes, and 72°C for three minutes which resulted in the amplification of a 459 base pair DNA product as determined by analysis of five microliters of the reaction sample on a ethidium bromide stained 0.7% TAE agarose gel. A

444 base pair product was purified using standard methods from a 1% TAE agarose gel after digestion of a sample of the 459 base pair amplification product with *Nco*I and *Eco*RI restriction endonucleases. The 444 base pair product was ligated into compatible sites in pMON7259 to generate pMON15028. Cell lysates prepared as above from IPTG induced cultures of JM101 containing pMON15028 were analyzed for the presence of AMPA acyltransferase activity and compared to cultures containing pMON15027. The results were indistinguishable, thus confirming that *phn*O encoded an enzyme capable of AMPA acylation. In addition, this result indicated that the P2A mutation in the protein, which was introduced into the gene coding sequence as a result of thermal amplification using the AATPCR6 oligonucleotide primer (SEQ ID NO: 5), was without effect on the acyltransferase activity of the resulting PhnO protein when expressed in *E. coli*.

Example 5

This example illustrates the production of polyclonal antibodies directed to the PhnO peptide.

Further studies of the *phn*O gene product required the use of antibodies directed to the PhnO protein. Therefore, PhnO was overproduced in *E. coli* JM101for for use as an immunogen in stimulating the production of antibodies upon injection into a goat. The *phn*O gene containing the P2A mutation in plasmid pMON15028 was introduced into plasmid pMON17061 on an *Nco*I to *Eco*RI DNA fragment, producing pMON15032. *phn*O expression in pMON15032 is under the control of the *E. coli rec*A promoter adjacent to the bacteriophage T7 gene 10L ribosome binding sequence. Cells were grown to mid log phase and induced by addition of nalidixic acid to the culture to approximately 50 parts per million, from a stock solution of 50 mg nalidixic acid powder dissolved in 1 ml 0.1 N NaOH. The culture was maintained under inducing conditions for twelve hours at 37°C. Cells were harvested as described in example 3, and sonicated in phosphate buffered saline. About 23% of the total soluble protein in the induced *E. coli* lysates was determined to be PhnO and approximately 60% of the total PhnO protein was released into the soluble phase as judged by SDS-PAGE and Coomassie blue staining. The protein was further purified by preparative SDS-PAGE providing a sufficient quantity of PhnO for use in producing antibody which binds to or reacts antigenically with PhnO or related AMPA transacylase proteins. Briefly, the PhnO protein was separated by size from other proteins in a 15% SDS-PAGE gel. A gel slice containing the PhnO protein was excised, weighed, and

homogenized using a polytron in a volume of phosphate buffered saline (PBS, pH 7.0) equal to the mass of the gel slice. The homogenate was mixed with an equal volume of complete Freund's media until a colloidal mixture was obtained. An 8-ml inoculum of this mixture was used for the first injection into a goat. Two weeks post-injection, a 50-ml bleed was collected and serum was separated from blood solids by centrifugation. A booster injection of gel purified PhnO protein was administered in a colloidal mixture of 50% incomplete Freund's adjuvant at four weeks, and at six weeks a second bleed was obtained.

The serum from the second bleed was used to screen for the presence of sufficient antibody titers specific for PhnO protein. Extracts from JM101 cells containing pMON15032 were subjected to western blot analysis. The concentration of protein in the extract was determined to be about 55 mg/ml by Bradford assay, and a prior Coomassie stained gel using this same extract was subjected to a densitometer scan which indicated that about 23% of the total cell protein was PhnO. The extract was desalted over a PD10 column, eluted with 10 mM Tris pH 7.5, and diluted with an equal volume of 2X SDS sample buffer. Serial dilutions were prepared using 1X sample buffer and loaded into wells of a 15% SDS PAGE gel. Additional samples were mixed with a tobacco leaf protein extract containing 10 additional micrograms of protein per lane in addition to the *E. coli* PhnO extracts. The tobacco leaf protein extracts were used to screen for the presence of cross reactive antibody to plant proteins. Proteins were separated according to size by electrophoresis at 7.5 mA constant for fourteen hours at 4°C, and the gel was electroblotted onto a MSI 0.45 micron nitrocellulose filter at 0.5 Ampere in Tris-Glycine transfer buffer for one hour. The membrane was then blocked with TBST (Tris, BSA, NaCl, Tween-20, Short Protocols in Molecular Biology, 3rd Ed., Wiley and Sons, Pub.) for two hours at room temperature, incubated forty-five minutes with a 1:500 dilution of the second bleed serum at room temperature, washed two times in TBST, incubated another forty-five minutes with alkaline phosphatase conjugated rabbit anti-goat IgG (Boehringer Mannheim Biochemicals, Inc.), washed three times with TBST and one time with alkaline phosphatase buffer, and finally incubated for two and one half minutes with a standard color development solution containing NBT and BCIP. The reaction was terminated by washing the membrane with ample quantities of distilled water. The antibody was able to detect PhnO protein in as little as 50 nanograms of *E. coli* extract independent of the presence of additional plant proteins in one half of the samples. In addition, very few cross reactive bands were detected in either set of

samples, indicating that the serum sample contains very little IgG which cross reacts with either *E. coli* or tobacco plant proteins when tested using this western blot method.

An alternative source for generating antibody which is capable of specific binding to or reacting antigenically with PhnO protein was also utilized. A *phn*O gene was placed into a commercial vector (Invitrogen) containing a metal binding amino acid coding sequence (His6) upstream of and in-frame with the *phn*O coding sequence. The His6-*phn*O DNA sequence was inserted into the *E. coli* expression vector pMON6235 on an *Nco*I to *Eco*RI fragment, under the control of an *E. coli* arabinose operon *ara*BAD promoter, producing plasmid pMON32909. His6-PhnO protein was produced upon arabinose induction of *E. coli* W3110 cells containing pMON32909, and purified over a metal affinity column according to the manufacturers' instructions.

His-tagged purified His6-PhnO protein standard was injected into 6 New Zealand White rabbits using an immunization procedure similar to that used for the goat, described above. Antiserum raised in these rabbits was also shown to be specific for binding PhnO protein and non-cross reactive with other *E. coli* bacterial or tobacco plant proteins.

Example 6

This example illustrates properties of an AMPA transacylase enzyme using aminomethylphosphonate and acetyl-CoA as substrates in an enzyme assay as measured by endpoint kinetic analysis.

The apparent Km (Km) and Vmax (Vmax) of PhnO enzyme were determined for the substrates aminomethlyphosphonate and acetyl-CoA. Determination of the PhnO Km and Vmax were made by endpoint kinetic analyses, determining the enzyme velocity in consuming each substrate at varying substrate concentrations, and plotting the inverse of the enzyme velocity versus the inverse of the substrate concentration to produce a Lineweaver-Burk plot of enzyme kinetics. The conversion of [^{14}C]-AMPA to N-acetyl-[^{14}C]-AMPA was monitored as in example 2, using enzyme in a desalted crude lysate of *E. coli* expressing *phn*O from pMON15032, produced as in example 4. Total protein per ml of extract was determined by the method of Bradford which indicated approximately 22.5 mg/ml. Densitometric scanning of Coomassie stained SDS-polyacrylamide gels resolving PhnO protein from these lysates indicated that PhnO represents about 23% of total protein, thus the cell extract was determined to contain about 5.2 mg PhnO protein per ml. In a first assay to determine the apparent Km and Vmax of

PhnO for AMPA, [^{14}C]-AMPA concentrations ranged from 2 to 38 mM. Enzyme reactions were incubated at 37°C for 5 minutes and quenched with 1 volume of 100 mM sodium acetate (NaOAc), pH 4.4, in ethanol. Samples were analyzed by HPLC to determine the amount of [^{14}C]-AMPA converted to N-acetyl-[^{14}C]-AMPA. The assay conditions and output for each set of reactions are shown in Table 8.

Table 8.

PhnO Enzyme Kinetics for AMPA Substrate

Sample	S[1]	%Turnover[2]	Velocity[3]	1/S	1/V	V/S
1	200	39.5	79	1.0	0.0127	79.00
2	400	35.1	140	0.5	0.0071	70.00
3	800	32.9	263	0.25	0.0038	65.75
4	1200	26.8	322	0.166	0.0031	53.67
5	1600	26.2	426	0.125	0.0023	53.25
6	2000	22.1	442	0.100	0.0023	44.20
7	2400	19.2	461	0.083	0.0022	38.42
8	2800	17.6	493	0.071	0.0020	35.21
9	3200	17.3	554	0.063	0.0018	34.63
10	3600	14.5	522	0.056	0.0019	29.00
11	4000	13.6	544	0.050	0.0018	27.20
12	6000	12.7	762	0.033	0.0013	25.15
13	7600	10	760	0.026	0.0013	19.76

1 - AMPA substrate concentration in reaction in nm (nanomoles)
2 - % turnover measured by the percent of N-acetyl-[^{14}C]-AMPA formed in relation to the amount of [^{14}C]-AMPA remaining in the sample
3 - enzyme velocity in units of AMPA (nm) converted to N-acetyl-AMPA per minute per mg of protein

A Linweaver-Burk plot of the 1/V vs 1/S data from Table 8 indicates that the apparent Km of PhnO for AMPA as a substrate is about 9 mM, and the apparent Vmax is about 824 U/mg protein.

The apparent Km of PhnO for the substrate acetyl-CoA was determined in similar experiments. After several attempts to obtain end point kinetics, it was determined that the turnover number was too low to be reliable at AMPA concentrations of about 30 mM and enzyme amounts of about 1-10 ng. An alternative approach was tried using tritium labeled acetyl-CoA. The specific activity of the label was about 40 X higher than with [^{14}C], providing a gain in sensitivity that allowed for the determination of the apparent Km of PhnO for Acetyl-CoA. The [^{3}H]-acetyl-CoA (Amersham, Inc.) specific activity was 360 mCi/mg or 250 μCi/ml.

The transacylation mediated by PhnO from [^{3}H]-acetyl-CoA to [^{3}H]-acetyl-AMPA was monitored by weak anion exchange HPLC chromatography, with the retention times of acetyl-CoA and acetyl-AMPA adjusted so that these compounds were separated by about three minutes. This was accomplished by adjusting the concentration of KH_2PO_4 buffer (pH 5.5) to 40 mM with a flow rate of 1 ml per minute over an AX100 weak anion exchange column. Each sample was reacted with PhnO and 30 mM AMPA for five minutes at 37°C and quenched with 100 mM NaOAc pH 4.4 in ethanol, then analyzed by HPLC. [^{3}H]-acetyl-CoA substrate ranged from 25 micromolar to 1.3 mM in each reaction along with about 5ng PhnO, 50 mM Tris pH 7.5, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 30 mM AMPA. Samples were analyzed by HPLC to determine the amounts of N-[^{3}H]-acetyl-AMPA produced, and [^{3}H]-acetyl-CoA remaining. The assay conditions and results for these reactions are shown in Table 9.

Table 9.

PhnO Enzyme Kinetics for Acetyl-CoA Donor Substrate

Sample No.	[Acetyl-CoA][1]	Velocity[2]	1/[S][3]	1/V[4]	V/S[5]
1	25	34	0.0400	0.0294	1.3600
2	50	66	0.0200	0.0152	1.3200
3	75	94	0.0133	0.0106	1.2533
4	100	125	0.0100	0.0080	1.2500
5	125	150	0.0080	0.0067	1.2000
6	150	173	0.0066	0.0058	1.1533
7	175	193	0.0057	0.0052	1.1029
8	200	219	0.0050	0.0046	1.0950
9	225	240	0.0044	0.0042	1.0667
10	250	259	0.0040	0.0039	1.0360
11	375	339	0.0027	0.0030	0.9040
12	390	287	0.0026	0.0035	0.7359
13	520	331	0.0019	0.0030	0.6365
14	650	352	0.0015	0.0028	0.5415
15	780	372	0.0013	0.0027	0.4769
16	910	397	0.0011	0.0025	0.4363
17	1040	411	0.0009	0.0024	0.3952
18	1170	425	0.0008	0.0024	0.3632
19	1300	434	0.0007	0.0023	0.3338

1 - substrate concentration in micromolar units
2 - enzyme velocity as measured by amount of [^{3}H] incorporated into [^{3}H]-acetyl-AMPA per unit time
3 - inverse substrate concentration
4 - inverse velocity
5 - ratio of velocity to substrate concentration

A Linweaver-Burk plot of the 1/V vs 1/S data from Table 9 indicates that the apparent Km of PhnO for acetyl-CoA as a substrate is between 375-390 micromolar, and the apparent Vmax is about 824 U/mg protein.

An approximate pH range of activity for the PhnO enzyme was determined using enzyme in a crude lysate of *E. coli* expressing *phn*O from pMON15032. The ability of the enzyme to produce N-acetyl AMPA from a mixture containing acetyl-CoA and AMPA across a range of pH values was determined. The reactions were carried out in MES/MOPS/Tricine buffer equilibrated to a pH value from 4.5 to 9.0, with actual pH values ranging from 5.2 through 9.0. Briefly, 95 microliters of an appropriate buffer was mixed with 100 microliters of 2X assay mix as described in example 4, and 5 microliters of desalted *E. coli* lysate containing approximately 400 ng/microliter PhnO protein. The reaction was incubated at 37°C for five minutes and quenched with 100 mM NaOAc pH 4.4 in ethanol, and analyzed by HPLC as described in example 4. The results are shown in Table 10.

Table 10.

PhnO Enzyme pH Profile

Buffer pH	[1]Mock Reaction pH	[2]% Turnover	N-Acetyl CoA (nmole)	[3]Velocity (nmole/min/microgram)
5.0	5.23	3.7	222	22.2
5.5	5.62	3.9	234	23.4
6.0	5.92	4.2	252	25.2
6.5	6.47	13.3	798	79.8
7.0	7.0	27.0	1620	162.0
7.5	7.48	32.0	1920	192.0
8.0	8.05	34.3	2058	205.8
8.5	8.46	33.5	2010	201.0
9.0	9.0	33.9	2034	203.4

1- indicates true pH value after combining all reagents for each initial buffer pH value given
2- determined as in Table 9 for Km and Vmax
3- determined as in Table 9 for Vmax

The results indicate that optimum PhnO transacylase activity using AMPA and acetyl-CoA as substrates is about pH 8.0. However PhnO efficiently converts AMPA to N-acetyl-AMPA using acetyl-CoA as the acetyl donor across a pH range from about 6.5 to at least 9.0.

Additional experiments were carried out with purified PhnO protein to further characterize the scope of the enzyme's substrate preference for acyl-CoA acyl donor compounds. It has been established herein that at least one substrate acyl- donor or leaving group can be a

two carbon acid compound such as the acetyl- moiety in the compound Acetyl-CoA. It was not known what range of acyl- molecules comprised of different carbon chain lengths would or could function as a leaving group from the acyl-CoA acyl donor when reacted with PhnO transacylase and AMPA as the acyl- receptor molecule. Therefor, an HPLC assay similar to that described in Example 2 was developed to determine the scope of the enzymes' ability to transfer an acyl- group from an acyl-CoA acyl donor to [^{14}C]-AMPA.

PhnO was purified from a one liter Luria Bertani broth culture of *E. coli* JM101 expressing a recombinant *phn*O gene from pMON15032 after nalidixic acid induction for three hours at 37°C. Cells were harvested by centrifugation and resuspended in 40 ml cold Tris buffer (0.1 M Tris-HCl pH 8) and placed on ice. The cell suspension was brought to 1 mM DTT and 0.5 mM PMSF. The suspension was lysed by 2 passages through a prechilled French pressure cell at 1,100 psi, centrifuged at 12,000 g (10,000 rpm in an Sorvall SA600 rotor) for 40 min at 4°C, then placed on ice. The cleared supernatants were poured into fresh 15 ml polypropylene tubes. The samples were split again into two equal portions and maintained at -80°C until used further for purification of PhnO protein. 20 microliters of the soluble fraction was assayed for enzyme activity using the HPLC method described above in Example 2, except after terminating the assay with acid addition, the sample was stored at -80°C. A Sephacryl S200 column was prepared according to the manufacturers' instructions and equilibrated with a solution containing 20 mM Tris pH 8.0 and 0.5 mM $MgCl_2$. The entire total soluble extract was layered over the top of the column bed after thawing on ice. Forty 9 ml fractions were collected from the column eluate, and thirty microliters of each fraction was analyzed by western blot using anti-PhnO antiserum after resolution on a 15% SDS-PAGE gel. Also, thirty microliters of each fraction was analyzed for AMPA acyl transferase activity using the method described in Example 2. Samples which exhibited acyl transferase activity and which corresponded to positive western blot data were pooled. These were represented by fractions 7 through 19 in this example, and were combined into a 100 ml volume, distributed into ten 10 tubes each containing 10 ml volumes, and stored at -80°C for further use.

Anion exchange chromatography was used to determine the elution pattern of PhnO away from other contaminating proteins that co-elute during the Sephacryl S200 fractionation. One tube from the combined PhnO positive fractions was thawed on ice and injected into a 5/5 Mono-Q column pre-equilibrated with buffers A (one liter of 20 mM Tris-HCl pH 8.0 Mili-Q distilled

deionized water) and B (one liter of 20 mM Tris-HCl pH 8.0, 1 M NaCl). The sample containing PhnO active protein was injected into the column and one milliliter fractions were collected. The column was washed for five minutes with a flow rate of 1.8 ml per minute Buffer A after loading the PhnO containing sample. At five minutes, Buffer B was added to the flow volume at 0.5 ml per minute for four minutes. Buffer B was ramped up to 22% of the flow volume at 10 minutes, 30% at 12 minutes, 36% at 13 minutes, 41% at 14 minutes, 46% at 15 minutes, 74% at 16 minutes, and 100% at 16 minutes through 22 minutes, at which point Buffer B flow was terminated and Buffer A was reinitiated at 100% to equilibrate the column. Ten microliter volumes from individual fractions collected from the Mono-Q column were analyzed by western blot and for transacylase activity as described in Example 2. Fractions which exhibited positive AMPA acyltransferase activity and which correlated with the Western blot data were pooled and maintained as a purified protein sample. Samples of this purified PhnO protein were used to determine enzyme's acyl donor substrate specificity.

Enzyme reactions were prepared as follows. 100 microliter reactions consisted of 50 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 3 microliters of 1.3 mM [^{14}C]-AMPA (115,392 dpm per microliter), 0.1 mM or 1 mM acyl-CoA acyl donor, and 2.5 microliter purified enzyme sample. A assay premix was prepared from which 45 microliters was used in each 100 microliter reaction. This 45 microliter premix sample consisted of 40 microliters distilled and deionized water, 2 microliters of 50 mM $MgCl_2$, and 3 microliters of 1.3 mM [^{14}C]-AMPA (115,392 dpm per microliter). Reactions were initiated by mixing 40 microliters of 125 mM Tris-HCl pH 8.0, 2.5 microliters protein sample and 10 microliters acyl-CoA acyl donor compound in a microcentrifuge tube at room temperature. Each acyl-CoA acyl donor compound was prepared as a stock solution of 1mM, 5 mM or 10 mM stocks. Each tube was then mixed with 45 microliters of the assay premix containing the [^{14}C]-AMPA receptor substrate, mixed gently and transferred to a 30°C water bath for 5 minutes. Each reaction was terminated with the addition of 4 microliters of 1M HCl, mixed by vortexing, and placed on ice or stored at -20°C until assayed for the presence of [^{14}C]-AMPA or related compounds by HPLC.

HPLC analysis was carried out using a Waters 510 dual pump HPLC system with a 481 wavelength max UV detector and a scintillation pump, a Phenomenex PHENOSPHERE 5 micrometer 80Å SAX-silica HPLC column (250X4.6 mm, 3500 PSI max pressure), Buffer A consisting of 5 mM KH_2PO_4, 4% methanol, adjusted to pH 2.0 with H_3PO_4, and Buffer B

consisting of 200 mM KH_2PO_4, 4% methanol adjusted to pH 2.0 with H_3PO_4, and HAZARD Atomflow (Packard) containing 64% 1,2,4 trimethylbenzene, 7.5% sodium-dicotyl sulfosuccinate, 3.5% sodium diamylsulfosuccinate, and 6% polyoxyethylene(4)lauryl ether. HPLC gradient conditions for each sample analysis were similar to those described in Example 2, with minor variations. The flow rates are provided in Table 11.

Table 11.

HPLC Gradient Conditions				
Time (min)	Flow (ml/min)	%A	%B	Flow Rate[1]
0.0	1	100	0	3
2.0	1	100	0	3
5.0	1	50	50	3
15.0	1	0	100	3
17.0	1	0	100	3
17.3	1	100	0	3
21.0	1	100	0	3
21.3	0.1	100	0	0

1- Scintillation fluid flow rate in milliliters per minute

Stock solutions of Acyl-CoA acyl donor compounds were prepared as described above, and these are listed here: Na Acetyl-CoA, Li n-propionyl-CoA, Li glutaryl-CoA, Li methylmalonyl CoA, Li crotonoyl-CoA, Li isobutyryl-CoA, Na succinyl-CoA, Li tiglyl-CoA, Li n-valeryl-CoA, and Li desulfo-CoA. All compounds were obtained from Sigma Chemical Company, St. Louis, MO. The percent activity of the purified enzyme for transfer of the CoA associated acyl- moiety to [^{14}C]-AMPA was determined by measuring the percentage of [^{14}C]-AMPA HPLC chromatogram peak area converted to some other [^{14}C]-compound, such as N-acetyl-[^{14}C]-AMPA, with the amount of N-acetyl-[^{14}C]-AMPA produced during the reaction in which [^{14}C]-AMPA and 1 mM acetyl-CoA are substrates for PhnO being established as the 100% reference. The results are shown in Table 12.

Table 12.

AMPA Transacylase Enzyme Efficiency for Acyl-CoA Acyl Donor Substrate

Acyl-CoA Acyl Donor	[^{14}C]-AMPA % Conversion[1]	% Activity
Acetyl-CoA 0.1 mM	79.2	79.2
Acety-CoA 0.5 mM	98.7	98.7
Acety-CoA 1 mM	100.00	100.00
Propionyl-CoA 0.1 mM	78.2	78.2
Propionyl-CoA 0.5 mM	97.8	97.8
Propionyl-CoA 1 mM	100.00	100.00
Glutaryl-CoA 0.1 mM	0.81	0.81
Glutaryl-CoA 0.5 mM	0.00	0.00
Glutaryl-CoA 1 mM	0.57	0.57
Methylmalonyl-CoA 0.1 mM	1.11	1.11
Methylmalonyl-CoA 0.5 mM	2.08	2.08
Methylmalonyl-CoA 1 mM	2.21	2.21
Crotonoyl-CoA 0.1 mM	0.80	0.80
Crotonoyl-CoA 0.5 mM	0.00	0.00
Crotonoyl-CoA 1 mM	0.00	0.00
Isobutyryl-CoA 0.1 mM	2.10	2.10
Isobutyryl-CoA 0.5 mM	0.20	0.20
Isobutyryl-CoA 1 mM	0.00	0.00
Succinyl-CoA 0.1 mM	5.06	5.06
Succinyl-CoA 0.5 mM	3.38	3.38
Succinyl-CoA 1 mM	1.56	1.56
Tiglyl-CoA 0.1 mM	0.00	0.00
Tiglyl-CoA 0.5 mM	0.00	0.00
Tiglyl-CoA 1 mM	0.99	0.99
Valeryl-CoA 0.1 mM	0.24	0.24
Valeryl-CoA 0.5 mM	0.00	0.00
Valeryl-CoA 1 mM	0.33	0.33

Table 12. (continued)

Acyl-CoA Acyl Donor	[^{14}C]-AMPA % Conversion[1]	% Activity
Desulfo-CoA 0.1 mM	0.95	0.95
Desulfo-CoA 0.5 mM	1.25	1.25
Desulfo-CoA 1 mM	0.52	0.52

1 - percentage of [^{14}C]-AMPA HPLC chromatogram peak area converted to some other [^{14}C]-compound, such as N-acetyl-[^{14}C]-AMPA, with the amount of N-acetyl-[^{14}C]-AMPA produced during the reaction in which [^{14}C]-AMPA and 1 mM acetyl-CoA are substrates for PhnO being established as the 100% reference

These results indicate that PhnO enzyme is capable of efficiently utilizing acyl-CoA associated compounds which have an acyl group with a carbon chain length of not more than three for transacylating AMPA. Other compounds which have a longer carbon chain length than

propionyl- and which are not broad or bulky, such as methylmalonly-, isobutyryl-, and succinyl -CoA compounds are also effective acyl-CoA acyl donors, but at a lower enzyme efficiency.

Example 7

This example illustrates the *in vitro* expression and targeting of an AMPA acyltransferase protein into isolated chloroplasts.

Many chloroplast-localized proteins are expressed from nuclear genes as precursors and are targeted to the chloroplast by a chloroplast transit peptide (CTP). The CTP is removed during steps involved in import of the targeted protein into the chloroplast. Examples of such chloroplast proteins include the small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase (RUBISCO), 5-enol-pyruvylshikimate-3-phosphate (EPSPS), ferredoxin, ferredoxin oxidoreductase, the light-harvesting-complex protein I and protein II, and thioredoxin F. It has been demonstrated *in vivo* and *in vitro* that non-chloroplast proteins may be targeted to the chloroplast by use of fusions with a CTP and that a CTP sequence is sufficient to target a protein to the chloroplast (Della-Cioppa et al., 1987). 5-enolpyruvylshikimate-3-phosphate synthetase (EPSPS) enzyme is located in the chloroplast and is the glyphosate target in plants. Targeting glyphosate oxidoreductase to the chloroplast has been found to provide tolerance to plants to glyphosate, although GOX localized to the cytoplasm is also able to provide such tolerance. Generally, recombinant GOX enzyme is localized to the chloroplast. GOX mediated glyphosate metabolism produces AMPA, which has been shown to be phytotoxic. It has been shown herein that PhnO is capable of AMPA N-acylation and that N-acetyl-AMPA is not phytotoxic. Therefore, it may be necessary to inactivate AMPA in plants. This assumes that AMPA acyltransferase can be expressed in plants as an active enzyme, and that such acyltransferases are capable of being imported into the chloroplast and retain enzymatic activity. In view of the AMPA phytotoxicity as described in example 1, an AMPA acyltransferase gene was introduced into plant expression vectors to test expression in plants. In addition, import of acyltransferase into chloroplasts was also tested.

A DNA sequence encoding a chloroplast targeting peptide was linked 5' to and in frame with a DNA sequence encoding an AMPA acyltransferase. A DNA sequence encoding an arabidopsis ribulose-1-bis-phosphate carboxylase small subunit chloroplast transit peptide (CTP, SEQ ID NO:9) was excised from pMON17058 using *Bgl*II and *Nco*I restriction endonucleases, and inserted into complementary restriction sites in pMON15028 to produce pMON15029, so

that the CTP coding sequence was linked 5' to and in frame with the *phn*O coding sequence in pMON15028. The resulting chimeric *phn*O gene in pMON15029 is capable of producing a chloroplast targeted PhnO protein. An *Eco*RI to *Bgl*II DNA cassette containing the CTP-PhnO coding sequence, SEQ ID NO:11, from pMON15029 was inserted into *Eco*RI and *Bam*HI sites in pBlueScript KS(-) to produce pMON15036. The CTP-PhnO coding sequence in pMON15036 can be expressed in an *in vitro* transcription/translation system from a phage T3 promoter. A similar plant transient expression plasmid, pMON15035, was constructed, but without the chloroplast targeting sequence. An *Eco*RI to *Bgl*II DNA fragment containing only the *phn*O coding sequence was excised from pMON15028 and inserted into *Eco*RI and *Bam*HI sites in pBlueScript KS(+) so that PhnO could be produced from a phage T7 promoter in an *in vitro* transcription/translation system. An *Nco*I to *Eco*RI DNA sequence encoding PhnO was excised from pMON15028 and inserted into pMON17061, producing pMON15032. pMON15032 provides for expression of *phn*O from an *E. coli rec*A promoter. A *Bgl*II to *Eco*RI DNA fragment encoding PhnO was excised from pMON15028 and inserted into pBlueScript SK(-) to produce pMON15033. pMON15033 provides for expression of *phn*O from an *E. coli lac* promoter. A *Bgl*II to *Eco*RI DNA fragment encoding CTP-PhnO was excised from pMON15029 and inserted into compatible sites in pBlueScript SK(-), providing for expression of chloroplast targeted PhnO protein from an *E. coli lac* promoter from pMON15034.

pMON15032, pMON15033, and pMON15034 were introduced into *E. coli* JM101. Cultures were grown and induced as described above, except that expression from cells containing pMON15032 was induced with addition of 50 parts per million nalidixic acid in 0.1 M NaOH. Cleared lysates were prepared from each culture and subjected to an AMPA acyltransferase assay as described above in order to determine the presence of AMPA acyltransferase activity. All lysates contained substantial amounts of acyltransferase activity above control levels. More importantly, the CTP-PhnO peptide (SEQ ID NO:12) expressed from pMON15034 appeared to retain full enzymatic acyltransferase activity.

pMON15035 (PhnO) and pMON15036 (CTP-PhnO) were used *in vitro* to generate [^{35}S]-methionine labeled PhnO protein for use in a chloroplast import assay. Briefly, the procedure used for *in vitro* transcription and translation was as described in *Short Protocols In Molecular Biology, Third Edition*, Ed. Ausubel et al., Wiley & Sons Pub., (1995), which is herein incorporated by reference. About 20 micrograms of plasmid DNA was digested to completion

with *Hind*III restriction endonuclease in a 100 microliter reaction. 20 microliters of the plasmid digest, or about 4 micrograms of linearized plasmid DNA, was used in an *in vitro* transcription reaction to generate mRNA for producing PhnO or CTP-PhnO protein product in later translation reactions. Transcription reactions consisted of 20 microliters of linearized plasmid DNA, 20 microliters of a 5X transcription buffer (200 mM TrisHCl pH 8.0, 40 mM $MgCl_2$, 10 mM spermidine and 250 mM NaCl), 20 microliters of 5X ribonucleoside triphosphate mix (5mm each ATP, CTP, UTP, 5 mM diguanosine triphosphate (G-5'ppp5'-G)TP, 5 mM GTP), 10 microliters 0.1 M dithiothreitol (DTT), 10 microliters RNasin™ (a pancreatic ribonuclease inhibitor mixture from Promega), 4 microliters RNA polymerase (T7 or T3, New England Biolabs, Inc.), and distilled, deionized water to 100 microliters. Each reaction was incubated at 37°C for one hour. 4.5 microliters of each reaction was analyzed on a 1.4% agarose formaldehyde gel to ensure that each reaction produced adequate RNA template for the following translation step.

20 microliters of the transcription reactions were used for producing [^{35}S]-methionine labeled PhnO proteins for use in a chloroplast import assay. Briefly, RNA was mixed with 6 microliters of an aqueous amino acid mixture without methionine, 15 microliters of [^{35}S]-methionine (1400 Ci/mmol, Amersham), and 200 microliters of a rabbit reticulocyte lysate. These reactions were incubated at 37°C for two hours and placed on dry ice for storage. A 10 microliter sample of each reaction was analyzed on a 15% SDS-PAGE gel. Gels were vacuum dried and placed directly onto the emulsion side of KODAK ™ X-O-MAT ™ film for autoradiography. The results indicated that each plasmid produced respective peptides of predicted molecular mass for PhnO (pMON15035) and CTP-PhnO (pMON15036) in sufficient quantity to test for uptake into chloroplasts in an import assay.

Intact chloroplasts were isolated from one head of deveined Romaine lettuce according to Edelman et al., *Methods in Chloroplast Molecular Biology*, Elsevier Biomedical Press, Chap. 86, 1982. One liter of grinding buffer (GR-buffer) stock was prepared (2 mM NaEDTA, 1 mM MgCl2, 1 mM MnCl2, 50 mM Hepes-KOH pH 7.5, and 0.33 mM sorbitol). Immediately before use, 890 mg of ascorbic acid was added to 900 ml of GR-buffer stock solution. One head of torn, deveined Romaine lettuce was mixed with 900 ml GR-buffer and emacerated by mixing in a Waring blender three times for three seconds each time at high speed. The slurry was filtered through four layers of Miracloth, and the filtrate was centrifuged at 5,000 RPM for 10 minutes at

4°C in a SORVALL™ GS-3 rotor. The supernatant was decanted and the pellet resuspended with a glass rod in 4 milliliters of GR-buffer. Chloroplasts were isolated by centrifugation through a Percoll gradient. 80% Percoll was prepared by mixing 16 mls of PBF-Percoll with 4 mls of 5X Buffer (10 mM EDTA, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 250 mM Hepes-KOH, 30 grams sorbitol, 490 mg NaAscorbate, 85.5 mg glutathione to 100 mls with ddH_20). A 40% Percoll solution was prepared by combining 8 mls PBF-Percoll with 4 mls 5X Buffer and 8 mls of ddH_20. A Percoll gradient was prepared in a 30 ml Corex tube by layering 10 mls of 40% Percoll onto 10 mls of 80% Percoll. Chloroplasts were isolated by layering the resuspended chloroplasts onto the percoll gradient, spinning at 9,500 RPM for ten minutes in an SS-34 SORVALL™ swinging bucket rotor at 4°C for ten minutes with the brake on. Broken chloroplasts remain in the upper layer and were pipetted off. The intact chloroplasts were located at the interface of the 40/80% Percoll gradient and were removed to a new 30 ml COREX™ tube. The isolated chloroplasts were washed two times with GR-buffer and centrifuged for collection after each wash in a SS-34 rotor at 6,000 RPM for ten minutes at 4°C with the brake off. Isolated, washed chloroplasts were resuspended in 1 ml sterile 50 mM Hepes-KOH pH 7.7, 330 mM sorbitol by gently stirring with a glass rod, and the chlorophyll concentration of the slurry was determined. 5 mls of an 80% acetone solution was added to 20 microliters of the chloroplast slurry and vortexed gently. The resulting mixture was filtered through a Whatman™ #1 filter paper into a culture tube. The absorbance of the filtrate was determined at 645nm and 663 nm against an 80% acetone blank. The chlorophyll concentration in micrograms per ml was determined according to equation #1 as [chlorophyll µg/ml] = [A_{645}+ [A_{663} * (8.02)]. The mass of the chlorophyll in µg is calculated by taking the amount of chlorophyll measured in µg/ml and multiplying by the volume into which the chloroplasts were resuspended (equation #2), which is 5 mls in this example. Thus, the concentration of chlorophyll in µg/µl in the measured sample is equivalent to the value determined in equation #2 divided by the volume of the sample measured, which in this example is 20 µl. In this example, A_{645} was determined to be 0.496, and A_{663} was determined to be 1.0814. Thus, the concentration of chlorophyll in the measured sample was 4.67 µg/µl. The concentration of chlorophyll in the chloroplast slurry was adjusted to 4.0 µg/µl with Hepes-KOH pH 7.7, 330 mM sorbitol solution and the resulting chloroplast suspension was stored on ice in the dark.

A typical 300 microliter uptake experiment contained 5 mM ATP, 8.3 mM unlabeled methionine, 322 mM sorbitol, 58.3 mM Hepes-KOH (pH 8.0), 50 microliters reticulocyte lysate translation products, and intact chloroplasts (about 200 microgram chlorophyll). The uptake mixtures were gently rocked at room temperature in 10X75 mm glass tubes, directly in front of a fiber optic illuminator set at maximum light intensity using a 150 Watt bulb. Two separate 70 microliter samples of each uptake mix were removed at 0, 5, 10 and 15 minutes. One sample was centrifuged over 100 microliter silicone-oil gradients in 150 microliter polyethylene tubes by centrifugation at 11,000 X g for 30 seconds, and immediately frozen in dry ice. Under these conditions, the intact chloroplasts form a pellet under the silicone-oil layer and the incubation medium containing the reticulocyte lysate remains floating on the surface of the interface. The other sample was treated with protease (one tenth volume or 7 microliters of 0.25 mg/ml each trypsin and chymotrypsin protease mixture) for thirty minutes on ice, then subjected to silicone-oil separation and frozen on dry ice. The chloroplast pellets were then resuspended in 50-100 microliters of a lysis buffer (10 mM Hepes-KOH pH 7.5, 1 mM PMSF, 1 mM benzamidine, 5 mM ε-amino-n-caproic acid, and 30 micrograms per ml aprotinin) and centrifuged at 15,000 X g for 20 minutes to pellet the thylakoid membranes. The cleared supernatant (stromal proteins) from this spin, and an aliquot of the reticulocyte lysate incubation medium from each uptake experiment, were mixed with an equal volume of 2X SDS-PAGE sample buffer and analyzed on a 15% SDS-PAGE gel, dried, and exposed to film as described above. Chloroplasts exposed to [^{35}S]-methionine labeled CTP-PhnO contained [^{35}S]-labeled protein of a size consistent with the predicted CTP- processed form of PhnO, while chloroplasts exposed to methionine labeled PhnO were devoid of labeled protein. Labeled protein imported into the chloroplasts was also protease resistant. These results indicated that PhnO could be targeted to chloroplasts when fused to a plastid targeting peptide sequence.

Example 8

This example illustrates the identification and characterization of plants transformed with an AMPA acyltransferase.

A wide variety of plant species have been successfully transformed using any number of plant transformation methodologies well known in the art. In particular, *Agrobacterium tumefaciens* mediated plant transformation is the preferred method presently in use, however, ballistic methods which increase delivery of naked DNA directly to plant cells through

microprojectile bombardment are also very effective in producing recombinantly transformed plants. In addition, methods which involve the use of liposomes, electroporation, chemicals that increase free DNA uptake, and transformation using viruses or pollen are alternatives which can be used to insert DNA constructs of this invention into plant cells. Plants which can be transformed by the practice of the present invention include but are not limited to corn, wheat, cotton, rice, soybean, sugarbeet, canola, flax, barley, oilseed rape, sunflower, potato, tobacco, tomato, alfalfa, lettuce, apple, poplar, pine, eucalyptus, acacia, poplar, sweetgum, radiata pine, loblolly pine, spruce, teak, alfalfa, clovers and other forage crops, turf grasses, oilpalm, sugarcane, banana, coffee, tea, cacao, apples, walnuts, almonds, grapes, peanuts, pulses, petunia, marigolds, vinca, begonias, geraniums, pansy, impatiens, oats, sorghum, and millet. DNA molecules for use in the present invention can be native or naturally occurring genes or chimeric genes constructed from useful polynucleotide sequences including promoters, enhancers, translated or non-translated leaders, sequences encoding signal peptides, sequences encoding transit peptides, structural genes, fusions of structural genes, terminators, introns, inverted repeats or direct repeats, linkers, and polyadenylation sequences. DNA sequences contemplated in this invention include single and double stranded polynucleotide sequences, linear sequences, and covalently closed circular polynucleotide sequences, plasmids, bacmids, cosmids, bacterial artificial chromosomes (BAC's), yeast artificial chromosomes (YAC's), and viral DNA and RNA sequences. In consideration of *Agrobacterium* mediated plant transformation, suitable plant transformation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens*, as well as those disclosed, for example by Herrera-Estrella (1983), Bevan (1984), Klee (1985) and EPO publication 120,516 (Schilperoort et al.). In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of *Agrobacterium*, alternative methods as described above can be used to insert the DNA constructs of this invention into plant cells.

Plasmids used for plant transformation generally were constructed from vectors which have been described elsewhere, particularly in US Pat No. 5,463,175 (Barry et al., 1995), which is herein incorporated by reference. Plasmids were constructed and maintained in *E. coli* using *Tn7* aminoglycoside adenylyltransferase resistance (*aad* gene, commonly referred to as streptomycin/spectinomycin or Spc/Str resistance), which is also a determinant for selection and maintenance in *Agrobacterium*. Other plasmid maintenance and selectable markers well known in the art for use in *E. coli* were also used, consisting essentially of neomycin

phosphotransferase, gentamycin acetyltransferase, and beta lactamase genes alone or present in combination on a single replicon or vector. Plasmids generally contain *ori*V, a replication origin derived from the broad host range plasmid RK2, and *ori*322 and *bom* (origin of replication for maintenance in *E. coli*, and basis of mobility for conjugational transfer respectively) sequences derived from plasmid pBR322.

A *phn*O gene encoding an AMPA acyltransferase was inserted into expression cassettes in plant transformation vectors. These cassettes generally contain the following elements in sequential 5' to 3' order: a sequence comprising a plant operable promoter, a sequence encoding a chloroplast or plastid transit peptide, a cloning site or sites contained within a polylinker, and a plant functional 3' nontranslated region. Expression cassettes often are constructed to contain unique restriction sites flanking the cassette domain so that the entire cassette can be excised from one plasmid and placed into other similarly constructed plasmid vectors. Restriction sites comprised of eight base pair recognition sequences are preferred, and most cassettes in the present invention are flanked at least on one end by a *Not*I restriction endonuclease recognition site. Preferred promoters are the figwort mosaic virus promoter, P-FMV (Gowda et al., 1989), the cauliflower mosaic virus 35S promoter CaMV 35S (Odell et al., 1985), or the enhanced CaMV 35S promoter (US Pat. No. 5,196,525; Kay et al., 1987). A number of other promoters which are active in plant cells have been described in the literature. Such promoters may be obtained from plants or plant viruses and include, but are not limited to the nopaline synthase (NOS) and octopine synthase (OCS) promoters which are carried on tumor-inducing plasmids generally found within virulent and non-virulent strains of *Agrobacterium tumefaciens*, the cauliflower mosaic virus (CaMV) 19S promoter, the comalina yellow mottle virus promoter, the sugar cane bacilliform DNA virus promoter, the peanut chlorotic streak virus promoter, the rice actin promoter, and the light-inducible ribulose 1,5-bisphosphate carboxylase small subunit promoter (ssRUBISCO). These promoters can used to create various types of DNA constructs useful for gene expression in plants (see for example Barry et al. US Patent No. 5,463,175). Particularly desirable promoters which are contemplated because of their constitutive nature are the Cauliflower Mosaic Virus 35S (CaMV35S) and the Figwort Mosaic Virus 35S (FMV35S) promoters which have previously been shown to produce high levels of expression in most plant organs. Other promoters which would direct tissue specific or targeted expression are also contemplated, for example in tissue such as leaves, meristem, flower, fruit and organs of

reproductive character. In addition, chimeric promoters are also envisioned. Nopaline synthase gene (NOS 3') and the pea ribulose bisphosphate carboxylase synthase E9 gene (E9 3') 3' nontranslated termination and polyadenylation sequences were also used.

Expression cassettes consisting of a AMPA acyltransferase structural gene inserted downstream of a promoter and between a sequence encoding a chloroplast targeting peptide and a 3' nontranslated sequence were generally present on a plant transformation vector. Expression cassettes were generally flanked on either end of the cassette by a nopaline type T-DNA right border region on one end and a left border region on the other end, both border regions derived from pTiT37 (Fraley et al., 1985). Some plant transformation vectors only contained the right border region, required for initiation of T-DNA transfer from *Agrobacterium* to the host cell. Most plant transformation vectors also contained a GOX (glyphosate oxidoreductase) gene, as described above, and in US Patent No. 5,463,175. GOX enzyme expressed from these vectors was generally targeted to the chloroplast when inserted into the plant genome.

Plant transformation vectors were mobilized into the ABI *Agrobacterium* strain A208 carrying the disarmed Ti plasmid pTiC58 (pMP90RK)(Koncz and Schell, 1986). The Ti plasmid does not carry the T-DNA phytohormone genes which induce crown gall formation. Mating of the plant vector into ABI was done by the triparental conjugation system using the helper plasmid pRK2013 (Ditta et al., 1980). Alternatively, the plant transformation plasmid can be introduced into the ABI strain by electroporation as described by Mattanovich et al. (*Efficient transformation of Agrobacterium spp. by electroporation.*, Nucleic Acids Res. (1989), 17(16), 6747), which is herein incorporated by reference. When plant tissue is incubated with the ABI::plant vector conjugate, the recombinant vector is transferred to the plant cells by the *vir* functions encoded by the disarmed pTiC58 plasmid. Ideally, the recombinant vector opens at the T-DNA right border region, and the DNA between the right and left border sequences is transferred directionally and inserted into the host plant genome, although the entire recombinant plant transformation vector sequence may be transferred and inserted. The pTiC58 Ti plasmid does not transfer to the plant cells but remains in the *Agrobacterium* donor.

Recombinant plants can be regenerated from plant cells or plant tissue which has been transformed with a functional AMPA acyltransferase structural gene. The choice of methodology for the regeneration step is not critical, with suitable protocols being available for hosts from Leguminosae (alfalfa, soybean, clover, etc.), Umbelliferae (carrot, celery, parsnip),

Cruciferae (cabbage, radish, rapeseed, etc.), Cucurbitaceae (melons and cucumber), Gramineae (wheat, rice, corn, etc.), Solanaceae (potato, tobacco, tomato, peppers), and various floral crops. See for example, Ammirato, 1984; Shimamoto, 1989; Fromm, 1990; and Vasil, 1990). Recombinant plants which have been transformed with an AMPA acyltransferase can also be selected on medium containing AMPA. The appropriate inhibitory concentration of AMPA can readily be determined by one of ordinary skill in the art for any particular host by screening for AMPA toxicity as described in example 1. Alternatively, when AMPA acyltransferase is transformed into plants previously transformed with GOX and selected for growth on glyphosate, either AMPA or glyphosate can be used as the selective ingredient for selecting for transformation events which express sufficient levels of AMPA acyltransferase enzyme. Glyphosate must be applied at levels which would otherwise be inhibitory to a recombinant plant expressing GOX and selected for growth on glyphosate, due to the increased level of AMPA which may be produced as a result of GOX mediated glyphosate degradation. In plants which express recombinant GOX enzyme, exposure to increasing levels of glyphosate has been shown to induce yellowing or chlorosis of the leaves, stunted growth characteristics, and infertility. AMPA acyltransferase expressed coordinately or in combination with GOX expression can overcome these detrimental effects. It is also possible to use AMPA as a plant transformation selectable marker as an alternative to glyphosate selection.

Tobacco

Tobacco plants were transformed with a *phn*O gene. A tobacco leaf disc transformation procedure employed healthy tissue from a leaf of about one month old. After a 15-20 minute surface sterilization with 10% CLOROX™ plus a surfactant, leaves were rinsed three times in sterile water. Leaf discs were punched with a sterile paper punch, and placed upside down on MS104 media (4.3 g/l MS salts, 30 g/l sucrose, 2 ml/l 500X B5 vitamins, 0.1 mg/l NAA, and 1.0 mg/l BA), and pre-cultured for one day. Discs were then inoculated with an 1:5 diluted overnight culture of disarmed *Agrobacterium* ABI containing the subject vector (final culture density about 0.6 OD as determined at 550 nm). The inoculation was done by placing the discs in sterile centrifuge tubes along with the culture. After thirty to sixty seconds, the liquid was drained off and the discs were blotted between sterile filter paper. The discs were then placed upside down on a filter disc on MS104 feeder plates and incubated for 2-3 days. After this co-culture period, the discs were transferred, still upside down, to selection plates containing MS104

media. After 2-3 weeks, callus formed, and individual clumps were separated from the leaf discs. Shoots were cleanly cut from the callus when they were large enough to distinguish from stems. The shoots were placed on hormone-free rooting media (MSO: 4.3 g/l MS salts, 30 g/l sucrose, and 2 ml/l 500X B5 vitamins) with selection. Roots formed in 1-2 weeks. Any leaf callus assays are preferably done on rooted shoots while still sterile. Rooted shoots were placed in soil and were maintained in a high humidity environment (ie: plastic containers or bags). The shoots were hardened off by gradually exposing them to ambient humidity conditions.

Three tobacco transformation events, designated as lines 33476, 36779, and 37235 were selected for further analysis. pMON17226 (Barry et al., US Patent No. 5,463,175, 1995) was used to produce plant line 33476 which contains an FMV-CTP-GOX gene construct. Lines 36779 and 37235 were produced using pMON17261, which is a plasmid derived from pMON17226 which contains *Not*I cassette containing an FMV-CTP-PhnO gene sequence (SEQ ID NO:11) in addition to FMV-CTP-GOX. The *Not*I cassette was constructed as follows. The sequence encoding CTP, represented by SEQ ID NO:9, was excised from pMON17058 as a *Bgl*II to *Nco*I fragment and inserted into pMON15028, forming a sequence represented by SEQ ID NO:11 in which the CTP coding sequence was upstream of and in frame with the PhnO coding sequence represented within SEQ ID NO:7. The resulting construct was designated as pMON15029. The CTP-PhnO coding sequence was excised from pMON15029 on a *Bgl*II to *Sac*I fragment and combined with pMON17063 fragments to produce pMON15038. pMON17063 was disassembled using restriction digestion to provide parts necessary for pMON15038 construction. pMON17063 was digested with *Sac*I and *Hind*III to produce a vector backbone into which a promoter fragment and the CTP-PhnO sequence were inserted. pMON17063 was also digested in a separate reaction with *Hind*III and *Bgl*II to produce a fragment containing an FMV promoter sequence. The promoter fragment and the CTP-PhnO fragment were ligated together in a reaction along with the vector backbone fragment to produce pMON15038, containing a *Not*I cassette harboring a sequence encoding a chloroplast targeted PhnO peptide expressed from an FMV promoter and flanked downstream by a NOS E9 3' transcription termination and polyadenylation sequence. This *Not*I sequence was excised from pMON15038 and inserted into the unique *Not*I site in pMON17241 to produce pMON17261, containing a chloroplast targeted GOX coding sequence expressed from an FMV promoter and flanked downstream by an E9 3' sequence, along with the CTP-PhnO coding sequence and

expression cassette. Transformation events derived from this vector are expected not only to be resistant to glyphosate, but to provide resistance to AMPA phytotoxicity as well. Lines 36779 and 37235 derived from pMON17261 were analyzed for the presence of genes encoding glyphosate oxidoreductase and AMPA acyltransferase by PCR, for the presence of GOX and PhnO enzymes by western blot, and for the presence of metabolites produced as a result of GOX mediated [^{14}C]-glyphosate degradation by HPLC.

Line 33476, obtained as a transformation event derived from pMON17226, was selected as a "GOX only" control. Lines 36779 and 37235 demonstrated different phenotypes upon exposure to glyphosate and were selected as glyphosate resistant events arising after transformation with pMON17261. Line 37235 became bleached or yellowed upon exposure to glyphosate, similar in phenotype to the GOX only line 33476. However, line 36779 displayed no such bleaching effect. DNA was extracted from leaf tissue for each of these events as well as from wt Samsun tobacco leaf, and subjected to PCR to determine the presence or absence of the transforming *phn*O gene.

Genomic DNA isolated from transformed tobacco lines was used as the template DNA in a PCR reaction and reaction products were compared to wild type Samsum tobacco. PCR reactions consisted of 50 microliters total volume containing 10X amplification buffer, 1.5 mM $MgCl_2$, deoxynucleotide mix with each at 1 mM, 50-100 ng genomic DNA, primers each at a final concentration of 16.8 pM, and 1.5 units of AmpliTaq DNA polymerase (Cetus/Perkin Elmer). Primers (synthesized to order by GENOSYS) consisted of the sequences as set forth in SEQ ID NO:21 and SEQ ID NO:22. SEQ ID NO:21 is a 20 base pair sequence capable of priming the synthesis of the P2A *phn*O gene sequence (SEQ ID NO:7) and hybridizes to the first twenty nucleotides of the coding sequence in that gene. SEQ ID NO:22 is also a 20 base pair sequence, but is capable of priming synthesis of a *phn*O gene from the terminal coding sequence into the structural coding region and hybridizes to the terminal twenty nucleotides of the sequence encoding PhnO. Amplification conditions consisted of three cycles of 97°C for one minute, 60°C for two minutes, and 72°C for two minutes, followed by 37 cycles of 94°C for one minute, 60°C for two minutes, and 72°C for two minutes, followed generally by a 4°C soak. 10 microliter samples were generally analyzed by 1% TAE agarose gel electrophoresis to resolve the relevant bands from residual primers. Upon ethidium bromide staining of the product gels, a *phn*O gene amplification product about 432 base pairs as judged by the migration position versus

*Hind*III digested lambda molecular weight markers appeared only in the line 33779 extracts, indicating the presence of the *phn*O gene in that line.

Seed from Ro transformation events were obtained after self crossing in growth chamber conditions. Ro seed were cured and planted to generate R1 progeny. Source leaves of R1 progeny at the five leaf stage were exposed to [^{14}C]-glyphosate by spotting a 2 microliter sample onto each vein(50 microliters of [^{14}C]-glyphosate Na+ salt, 517,000 dpm/microgram, 0.42 microgram/microliter mixed with 10 microliters of glycerol). Each leaf received several spots depending on the number of veins on that leaf. Three days later 15 additional 2 microliter spots were applied to each leaf. Two weeks later, five 2 microliter spots were applied to each of two leaves on each plant. These were new leaves and were not the older leaves to which glyphosate was initially applied. Five days after this last application, about 300 milligrams of tissue was sampled from two sink leaves on each plant. The samples from each plant were homogenized in separate 1ml volumes of deionized water, centrifuged at 9,000 RPM in a microcentrifuge, and the aqueous volumes were collected and stored on ice. Extracts were analyzed by HPLC for the presence of [^{14}C] labeled metabolites as in Example 2. The extract obtained from line 33476 (GOX) contained only [^{14}C]-AMPA. The extract obtained from line 37235 contained non-metabolized [^{14}C]-glyphosate as well as a trace but measurable amount of [^{14}C]-AMPA. Only N-acetyl-[^{14}C]-AMPA was observed in the extract obtained from line 36779. These results are consistent with the PCR data which indicated that line 36779 contained at least one copy of the *phn*O gene. In addition, the lack of a bleaching effect in line 36779 after exposure to glyphosate is consistent with the presence of functional GOX and PhnO enzymes and the absence of detectable [^{14}C]-AMPA.

Cotton

A recombinant *phn*O gene was transformed into Coker 312 variety cotton (*Gossypium hirsutum L.*). Glyphosate tolerant cotton lines were produced by *Agrobacterium* mediated plant transformation using double border binary plasmid vectors containing either (1) *gox*, an *Achromobacter sp.* strain LBAA gene encoding a glyphosate-metabolizing enzyme glyphosate oxidoreductase (GOX), (2) the *gox* gene and an *E. coli phn*O gene encoding PhnO, or (3) the *gox/phn*O double gene construct along with an *Agrobacterium* strain CP4 gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). All vectors are capable of replication in both *Agrobacterium tumefaciens* and *E. coli* hosts, and contain an aminoglycoside

adenylyltransferase gene (*aad*) conferring resistance to aminoglycosides such as spectinomycin or streptomycin and providing a method for plasmid maintenance.

pMON17241 contains a recombinant gene consisting of a 35S FMV promoter linked 5' to an *Arabidopsis thaliana* ribulose-1,5- bisphosphate carboxylase small subunit (SSU1A) gene sequence encoding a plastid or chloroplast targeting peptide (Timko et al., 1988) which is translationally fused to a *gox* gene coding sequence, which is linked 3' to a 3' untranslated region, designated E9, from a pea ribulose-1,5-bisphosphate carboxylase gene.

pMON17213 is a double gene plant transformation vector containing expression cassettes comprising (1) a 35S FMV promoter linked to a sequence encoding an *Arabidopsis thaliana* EPSPS chloroplast targeting peptide linked in-frame to a strain CP4 EPSPS coding sequence, which is linked 3' to an E9 3' untranslated region; and (2) a 35S FMV promoter linked to an SSU1A gene sequence encoding a plastid targeting peptide linked in-frame to a GOX coding sequence, which is linked 3' to a NOS 3' termination sequence.

pMON17261, described above, is a double gene plant transformation vector containing expression cassettes comprising (1) an FMV 35S promoter linked to an SSU1A chloroplast targeting peptide coding sequence linked in-frame to a GOX coding sequence, which is flanked downstream by the E9 3' untranslated region; and (2) an FMV 35S promoter linked to an SSU1A chloroplast targeting peptide coding sequence (SEQ ID NO:9) linked in-frame to a PhnO coding sequence (SEQ ID NO:7), which is linked 3' to a NOS 3' sequence.

pMON10151 is a double gene plant transformation vector containing expression cassettes comprising (1) an FMV 35S promoter linked to an SSU1A chloroplast targeting peptide coding sequence (SEQ ID NO:9) linked in-frame to a PhnO coding sequence (SEQ ID NO:7), which is flanked downstream by a NOS 3' sequence; and (2) an enhanced 35S promoter linked to an SSU1A chloroplast targeting peptide coding sequence linked in-frame to a GOX coding sequence which is flanked downstream by a NOS 3' sequence.

pMON10149 is a triple gene plant transformation vector containing expression cassettes comprising (1) an FMV 35S promoter and a petunia HSP70 5' untranslated leader sequence linked to an SSU1A chloroplast targeting peptide coding sequence linked in-frame to an EPSPS coding sequence, which is flanked downstream by the E9 3' termination and polyadenylation sequence; (2) an FMV 35S promoter linked to an SSU1A chloroplast targeting peptide coding sequence (SEQ ID NO:9) linked in-frame to a PhnO coding sequence (SEQ ID NO:7), which is

flanked downstream by a NOS 3' sequence; and (3) an enhanced 35S CaMV promoter linked to an SSU1A chloroplast targeting peptide coding sequence linked in-frame to a GOX coding sequence, which is flanked downstream by a nopaline synthase 3' polyadenylation sequence (NOS 3').

Plasmid vectors were assembled in *E. coli* K12 strains and mated into a disarmed ABI *Agrobacterium* strain. Aminoglycoside resistant *Agrobacterium* strains were used to transform Coker 312 derived hypocotyl sections with modifications as described by Umbeck et al. (1987) and Umbeck (US Patent No. 5,159,135 (1992), incorporated herein by reference), except that plants were regenerated with modifications described by Trolinder and Goodin (1987). Selection for glyphosate resistance produced several lines of cotton callus, which were subsequently determined by PCR of genomic DNA to contain the respective genes encoding EPSPS, GOX or PhnO transferred from *Agrobacterium*. Additionally, these same callus lines were determined by Western blot analysis to express the desired genes. After plant regeneration, whole cotton plants which contained the indicated coding sequences were recovered.

Previously identified plants transformed with a double gene glyphosate resistance cassette comprised of EPSPS and GOX encoding genes were determined to be resistant to glyphosate when applied at 48 ounces per acre through the 6-7 leaf stage, however severe bleaching of the leaves was observed. This phytotoxic effect was presumed to be due to the formation of AMPA as a result of GOX mediated glyphosate degradation. To test this, AMPA was sprayed at three different rates onto wild type Coker 312 plants. Leaf chlorosis and stunted growth was observed in plants at four days post-application of glyphosate at 640 ounces per acre and at eight days post-application of 64 ounces per acre. These results suggested that the phytotoxic effect observed in EPSPS/GOX transformed cotton plant lines was a result of GOX mediated AMPA production in plants, and that the phytotoxic effect may be obviated by co-expression of an AMPA acyltransferase along with GOX. To test this, cotton plants expressing GOX or GOX plus EPSPS alone or in combination with PhnO expression were treated with [^{14}C]-glyphosate, and the metabolism of the isotope labeled glyphosate was monitored in leaf tissue seven days after application.

Coker 312 glyphosate resistant recombinant cotton line 4416 was selected as a glyphosate resistant cotton line after transformation with pMON10149, a triple gene *Agrobacterium tumefaciens* mediated double border plant transformation vector containing chloroplast targeted

EPSPS, GOX, and PhnO, each expressed independently from separate 35S promoters. Several 4416 R3 plants were raised from R2 seed. One leaf of each plantlet at the three or four stage was treated with a mixture of ROUNDUP ULTRA™ commercial herbicide mixture (Lot No. GLP-9701-7428-F) which had been fortified with [^{14}C]-glyphosate (Code No. C-2251). The ROUNDUP ULTRA™ was shown to be 30.25% glyphosate acid by weight and the [^{14}C]-glyphosate had a radiochemical purity of 97.3% and a specific activity of 36.36 mCi/mmol. The treatment solution consisted of approximately 38 µL containing 1.60 x 10^6 dpm with a [^{14}C]-glyphosate specific activity of 1.713 x 10^3 dpm/µg glyphosate acid. Three or seven days after topical application the treated leaves were rinsed with water, frozen in liquid nitrogen, fractured with a spatula and then ground using a TEKMAR™ tissuemizer in 10 mL of water. The leaf extracts were adjusted to pH 3.5 - 4.0 with 1N HCl and approximately 4-8000 dpm were analyzed for the presence of [^{14}C]-metabolites by HPLC with liquid scintillation vial collection and detection (HPLC/LSC) as described in example 2. The new growth including the meristem and new leaves that emerged following topical application were also extracted and analyzed for [^{14}C]-metabolites. The results are shown in Table 13.

Table 13. [^{14}C]-Glyphosate Metabolism In Glyphosate Resistant Cotton

Line 4416 Plant#	% [^{14}C] metabolite in Glyphosate Treated Leaf Extract...*			% [^{14}C] metabolite in New Growth Extract...*		
	Glyphosate	AMPA	N-Acetyl-AMPA	Glyphosate	AMPA	N-Acetyl-AMPA
MD03	55.2	2.5	37.4	nd**	nd	93.4
MD04	94.6	2.1	1.7	97.9	nd	nd
A01	48.6	2.1	44.7	0.9	0.2	95.8
A02	67.3	2.0	29.1	0.7	0.2	96.5
A03	48.8	2.0	43.4	1.2	nd	94.0
A04	19.4	1.6	73.9	1.5	nd	94.0
A05	59.9	2.2	31.1	2.2	0.2	95.2
A06	38.2	nd	60.9	1.5	0.2	93.5
A07	64.1	nd	26.8	1.4	0.5	93.9
A08	90.9	2.0	1.9	91.2	2.5	1.9

* [^{14}C]-Glyphosate, [^{14}C]-AMPA, and N-Acetyl-[^{14}C]-AMPA as a percentage of total [^{14}C] isotope observed by HPLC/LSC in each sample.

** nd indicates that the metabolite was not detected by HPLC/LSC

Analysis of the water rinsed glyphosate treated leaves indicated the presence of significant levels of N-acetyl-[^{14}C]-AMPA in eight of the ten plants tested. These levels

represented 27-74% of the isotope extracted from the treated leaves The remaining activity was almost entirely [^{14}C]-glyphosate. Very little of the [^{14}C] isotope was present as [^{14}C]-AMPA. The remaining two plants had very limited ability to metabolize glyphosate as indicated by the high levels of [^{14}C]-glyphosate remaining on or in the leaves. One of these plants also showed signs of stunting seven days after treatment, indicating glyphosate phytotoxicity.

Analysis of new growth in the ten plants tested showed that the predominant form of [^{14}C] labeled metabolite present was N-acetyl-[^{14}C]-AMPA at greater than 90% of the total radioisotope in the samples. In contrast, more than 90% of the isotope in the remaining two plants was in the form of [^{14}C]-glyphosate, consistent with the analysis of the extract from the treatment leaf for these two plants.

The metabolism of [^{14}C]-glyphosate in recombinant cotton lines 4268 (GOX/PhnO) and 3753 (EPSPS/GOX) was also studied. Plants in this study were treated as indicated above for cotton line 4416, by applying droplets of ROUNDUP ULTRA fortified with [^{14}C]-glyphosate to a single leaf on each plant at the three to four leaf stage. Treated leaves were harvested and rinsed with water, then ground and extracted, and extracts were analyzed by HPLC as described above for the presence of [^{14}C]-glyphosate, [^{14}C]-AMPA, and N-acetyl-[^{14}C]-AMPA. New growth, including the meristem and new leaves that emerged following application were also extracted and analyzed. The results are shown in Table 14.

Table 14. [^{14}C]-Glyphosate Metabolism In Glyphosate Resistant Cotton

	*% [^{14}C] metabolite in Glyphosate Treated Leaf Extract...			*% [^{14}C] metabolite in New Growth Extract...		
Plant	Glyphosate	AMPA	N-Acetyl-AMPA	Glyphosate	AMPA	N-Acetyl-AMPA
GOX/PhnO Plants						
B01	76.7	3.0	14.0	3.4	1.0	89.9
B02	63.9	4.8	25.0	1.1	1.5	91.5
B03	54.4	3.2	36.4	0.8	nd	94.7
B04	58.3	5.7	28.9	1.1	1.2	91.0
EPSPS/GOX Plants						
C01	59.8	26.6	nd	3.72	85.7	nd
C02	92.7	2.1	0.8	92.8	0.8	nd
C03	81.2	10.7	nd	13.5	72.0	1.9
C04	86.2	6.4	1.0	13.9	76.2	nd

* [^{14}C]-Glyphosate, [^{14}C]-AMPA, and N-Acetyl-[^{14}C]-AMPA as a percentage of total [^{14}C] isotope labeled metabolites observed after HPLC/LSC analysis in each sample.
** nd indicates that the metabolite was not detected by HPLC/LSC.

Significant levels of N-acetyl-[^{14}C]-AMPA were present in the treated leaves of all four line 4268 plants (GOX/PhnO; B01-B04). In contrast, N-acetyl-[^{14}C]-AMPA was not detectable in extracts obtained from line 3753 plants (EPSPS/GOX; C01-C04). Three of these plants contained significant levels of [^{14}C]-AMPA in treated leaf extracts, ranging from 6-27%. One line 3753 plant was deficient in the conversion of [^{14}C]-glyphosate to N-acetyl-[^{14}C]-AMPA, and this plant also appeared to be stunted.

90-95% of the [^{14}C] isotope in extracts of new growth from line 4268 plants was determined to be in the form of N-acetyl-[^{14}C]-AMPA. However, 72-86% of the [^{14}C] isotope in extracts of new growth from three of the line 3753 plants was determined to be [^{14}C]-AMPA, with [^{14}C]-glyphosate accounting for the remainder of the isotope in these tissues. 93% of the isotope obtained from line 3753 plant number C02 was determined to be [^{14}C]-glyphosate, consistent with the lack of glyphosate metabolism in the application leaf as well as the observed stunting. In addition, growth regions of all line 3753 plants were discolored and yellow following treatment, but improved with time. By harvest, new growth leaves became mottled.

These results are consistent with the presence of active *gox* and *phn*O gene products in the indicated plants. The GOX and PhnO proteins are metabolizing glyphosate to AMPA and N-acetyl-AMPA in the predicted manner, and line 4268 plant extracts provide a similar metabolic pattern to that observed with line 4416 plant extracts as judged by HPLC and by phenotypic observation. In both lines, the predominant [^{14}C] product in new growth tissue extracts after [^{14}C]-glyphosate application is N-acetyl-[^{14}C]-AMPA. The phytotoxicity as observed by discoloration of plant leaves in line 3753 after glyphosate application is associated with the lack of an AMPA N-acyltransferase activity. In contrast, the presence of an AMPA N-acyltransferase activity in both the 4416 and the 4268 plant lines resulted in a lack of phytotoxic effects observed in line 3753 plants.

Canola

Canola plants were transformed with the vectors pMON17138 and pMON17261 and a number of plant lines of the transformed canola were obtained which exhibited glyphosate tolerance. Plants were transformed according to the method described in Barry et al. (US Pat No. 5,633,435). Briefly, *Brassica napus* cv Westar plants were grown in controlled growth chamber conditions as described. Four terminal internodes from plants just prior to bolting or plants in the process of bolting but before flowering were removed and surface sterilized in 70%

v/v ethanol for one minute, then in 2% w/v sodium hypochlorite for twenty minutes, then rinsed three times with sterile distilled deionized water. Stems with leaves attached could be refrigerated in moist plastic bags for up to three days prior to sterilization. Six to seven stem segments were cut into 5 mm discs with a Redco Vegetable Slicer 200 maintaining orientation of basal end. Stem discs (explants) were inoculated with 1 milliliter of ABI *Agrobacterium tumefaciens* strain A208 containing a recombinant plant transformation plasmid prepared as described above. Explants were placed basal side down in petri plates containing 0.1 X standard MS salts, B5 vitamins, 3% sucrose, 0.8% agar, pH 5.7, 1 mg/l BA (6-benzyladenine). The plates were layered with 1.5 ml of media containing MS salts, B5 vitamins, 3% sucrose, pH 5.7, 4 mg/l p-chlorophenoxyacetic acid, 0.005 mg/l kinetin and covered with sterile filter paper.

Following a 2.3 day co-culture, explants were transferred to deep dish petri plates (seven explants per plate) containing MS salts, B5 vitamins, 3% sucrose, 0.8% agar, pH 5.7, 1 mg/l BA, 500 mg/l carbenicillin, 50 mg/l cefotaxime, 200 mg/l kanamycin or 175 mg/l gentamicin for selection, and transferred after three weeks to fresh media, five explants per plate. Explants were cultured in a growth room at 25°C with continuous light (Cool White). After an additional three weeks, shoots were excised from the explants, and leaf recallusing assays were initiated to confirm modification of R_0 shoots. Three tiny pieces of leaf tissue were placed on recallusing media containing MS salts, B5 vitamins, 3% sucrose, 0.8% agar, pH 5.7, 5 mg/l BA, 0.5 mg/l naphthalene acetic acid (NAA), 500 mg/l carbenicillin, 50 mg/l cefotaxime, 200 mg/l kanamycin or gentamicin or 0.5 mM glyphosate. The leaf assays were incubated in a growth room under the same conditions as explant culture. After an additional three weeks, the leaf recallusing assays were scored for herbicide tolerance (callus or green leaf tissue) or sensitivity (bleaching).

Each shoot stem was dipped in ROOTONE at the time of excision, placed in a two inch pot containing Metro-MIX 350, and maintained in a closed humid environment in a growth chamber at 24°C, 16/8 hour photoperiod, 400 uE per square meter per second (HID lamps) for a hardening-off period of approximately three weeks.

Plasmid pMON17138 is an *Agrobacterium* mediated single border plant transformation vector maintained in the bacterium by selection on streptomycin or spectinomycin. pMON17138 contains a single right Ti border flanking the 3' end of the genetic elements desired to be transferred into the plant genome. This vector contains two plant operable expression cassettes. One cassette is comprised of a caulimovirus 35S promoter driving expression of a neomycin

phosphotransferase gene (*npt*II), flanked downstream by a nopaline synthase 3' transcription termination and polyadenylation sequence (NOS 3'). The other cassette is comprised of a figwort mosaic virus promoter (described in Rogers, US Pat No. 5,678,319) upstream of a pea ribulose bisphosphate carboxylase small subunit transcription termination and polyadenylation sequence. A chloroplast targeted glyphosate oxidoreductase (GOX) coding sequence is inserted between the promoter and pea 3' sequence.

Plasmid pMON17261 is an *Agrobacterium* mediated double border plant transformation vector similar to pMON17138. A chloroplast targeted GOX encoding cassette identical to that in pMON17138 is present downstream from a Ti right border, and upstream of an additional plant operable expression cassette comprised of a figwort mosaic virus promoter (P-FMV) linked to a NOS 3' sequence. A chloroplast targeted PhnO coding sequence is inserted between the second P-FMV and NOS3' sequences.

R_1 plants derived from transformation events using pMON17261 and pMON17138 were evaluated using a glyphosate spray test described in Barry et al. (US Pat No. 5,633,435).

Corn

An AMPA acyltransferase gene has also been introduced into Black Mexican Sweet corn cells with expression of the gene and glyphosate resistance detected in callus. Callus tissue was transformed according to the method described in Barry et al. (US Pat. No. 5,463,175). Various plasmids were used to introduce glyphosate resistance genes encoding GOX and EPSPS in combination with an AMPA acyltransferase gene into corn cells. These plasmids differed from each other with respect to promoters used, chloroplast or plastid targeting peptide sequences used, untranslated leader sequences used, presence or absence of an intron, and type of 3' terminator used, however all plasmids contained a synthetically derived AMPA acyltransferase gene encoding PhnO containing the P2A mutation. The synthetic gene was constructed from three smaller polynucleotide sequences synthesized for Monsanto and characterized for the presence of the desired DNA coding sequence and amino acid sequence translation by Stratagene, Inc., La Jolla, CA. The non-naturally occurring gene was assembled from three smaller sequences comprised of SEQ ID NO:16, SEQ ID NO:17, and SEQ ID NO:18, wherein the fully assembled gene is represented by SEQ ID NO:19, and is present in each of the plasmids used for the corn callus transformation. The non-naturally occurring gene coding sequence was established based on the method described in Fishhoff et al. in US Patent No. 5,500,365 in which

monocot preferred codons were used in place of those preferred by *E. coli*. The fully assembled gene encodes a full length PhnO protein identical to the native protein sequence with the exception of the P2A mutation introduced by PCR using SEQ ID NO:5 and SEQ ID NO:6 to engineer appropriate restriction endonuclease recognition sites into the flanking ends of the coding sequence. Plasmids which were used in generating the corn callus data are shown in Table 15 along with differences with respect to genetic elements flanking the AMPA acyltransferase encoding sequence.

Table 15. Corn Callus Transformation Plasmids and Relevant Genetic Elements

Plasmid	Relevant Genetic Elements*
pMON32926	[Pe35S / I-Zm.Hsp70 / CTP / phnO / T-At.Nos]→ GOX → EPSPS
pMON32931	[Pe35S / I-Zm.Hsp70 / phnO / T-At.Nos] → GOX → EPSPS
pMON32932	[Pe35S / I-Zm.Hsp70 / CTP / phnO / T-At.Nos] → GOX → EPSPS
pMON32936	[P-Os.Act1 / I-Os.Act1 / CTP / phnO / T-At.Nos]→ GOX → EPSPS
pMON32938	[P-Os.Act1 / I-Os.Act1 / CTP / phnO / T-At.Nos] → GOX → EPSPS
pMON32946	[Pe35S / L-Ta.Cab / CTP / phnO / T-Ta.Hsp70]]→ GOX → EPSPS
pMON32947	[Pe35S / L-Ta.Hsp70 / CTP / phnO / T-Ta.Hsp70] → GOX → EPSPS
pMON32948	EPSPS→ [Pe35S / I-Zm.Hsp70 / CTP / phnO / T-At.Nos] → GOX
pMON32950	EPSPS→[Pe35S / I-Zm.Hsp70 / CTP / phnO / T-At.Nos]→ GOX
pMON32570	EPSPS →[Pe35S / L-Ta.Cab / I-Os.Act1 / CTP / phnO / T-Ta.Hsp70]→GOX
pMON32571	EPSPS →[Pe35S / L-Ta.Cab / I-Os.Act1 / CTP / phnO / T-Ta.Hsp70] →GOX
pMON32572	EPSPS →[Pe35S / L-Zm.Hsp70 / I-Os.Act1 /CTP / phnO / T-Ta.Hsp70]→GOX
pMON32573	EPSPS →[Pe35S / L-Ta.Cab / I-Os.Act1 /CTP / phnO / T-Ta.Hsp70]→GOX

* Genetic elements contained within PhnO expression cassettes as indicated in each plasmid. Elements are shown in the order in which they appear in the plasmid, along with the presence of other genes encoding herbicide resistance, if present, flanking the PhnO expression cassette. → indicates the direction of transcription of each gene or genes flanking the PhnO expression cassette. Individual elements are described in the text.

Promoters which were used included the CaMV e35 S promoter and the rice actin promoter (P-Os.Act1). Introns which were used included those obtained from plant genes such as corn Hsp70 (I-Zm.Hsp70) and rice actin (I-Os.Act1). Non-translated leader sequences which were used included wheat chlorophyll a/b binding protein (L-Ta.Cab) and corn Hsp70 (L-Zm.Hsp70). Termination and polyadenylation sequences which were used included *Agrobacterium tumefaciens* NOS 3' (T-At.Nos) and wheat Hsp70 (T-Ta.Hsp70). The same chloroplast targeting sequence was used in all PhnO expression cassettes, represented by SEQ ID NO: 9.

A [^{14}C]-glyphosate metabolism assay was used for determining whether transformed corn callus tissues contain functioning forms of these enzymes. The assay was developed to screen large numbers of corn callus samples. Callus was obtained from Monsanto Company and

Dekalb Seed Company corn transformation groups. The Monsanto callus samples, individually designated as callus lines "19nn-nn-nn" in Table 16, were produced from HI II X B73 corn embryos. Callus samples were bombarded with complete covalently closed circular recombinant plant transformation vector plasmid DNA or with linear DNA fragments isolated from such plasmids 25–50 days after embryo isolation. Transformed lines were identified 8–14 weeks after bombardment. These lines were sub-cultured on fresh media every 2 weeks and were 5–7 months old when used in the metabolism assay. The Dekalb callus lines OO, OR, OW, OX, and OY were obtained from HI II x AW embryos. All line designations correspond to the recombinant plasmid or linear fragment used for ballistic transformation of callus tissue as noted in the legend to Table 16.

4.5 mCi of N-phosphono-[^{14}C]-methylglycine ([^{14}C]-glyphosate) was obtained from the Monsanto Radiosynthesis group in a 1.5 mM aqueous solution, having a specific radioactivity of 39.4 mCi/mM (5.2 X 10^5 dpm/microgram). The sample was identified with code number C-2182.2. A stock solution sterilized by filtration through a 0.2-micron Acrodisk (Gelman no. 4192) was prepared by combining 2.5 mL [^{14}C]-glyphosate (3.3 X 10^8 dpm) with 2.5 mL of corn callus growth medium (N6 medium) and 5.0 mg of Mon 0818 surfactant. [^{14}C]-glyphosate in the resulting dose solution was 0.75 mM. The N6 medium was described by Chu et al. (1975) and was prepared using salts and vitamins obtained from Sigma Chemical Company, St. Louis, MO. Mon 0818 surfactant is ethoxylated tallowamine, the surfactant used in Roundup herbicide. The dose solution was subjected to HPLC analysis as described in Example 2. The results are shown in a chromatogram illustrated in Figure 1. Three radioactive peaks were resolved, the largest of which corresponded to glyphosate (11.3 min, 98.8%). Impurity peaks corresponding to [^{14}C]-AMPA (5.8 min, 0.16%) and an unidentified material (10.2 min, 1.0%) were also present in the dose solution. No peaks corresponding to N-acetyl-[^{14}C]-AMPA were present in the dose solution. Two additional dose solutions were prepared using these reagents, each of which were scaled three fold to 15 ml volumes based on the preparation method described above.

N-acetyl-[^{14}C]-AMPA was synthesized for use as a retention time HPLC standard. 1 mL of pyridine and 2 mL of acetic anhydride was added to a 20-mL screw cap culture tube and chilled on ice. 0.1 mL of an aqueous solution of [^{14}C]-AMPA (6.2 X 10^6 dpm, code C-2127.2) was added to the chilled solution. The tube was then removed from the ice bath and warmed to

about 50–60°C. A 10-µL sample was removed after about 30 minutes and combined with 0.5 mL of water and analyzed according to the HPLC method set forth above. [^{14}C]-AMPA was not detected, however two new radioactive peaks were identified; one peak at 13.9 minutes (68%) and the other at 15.4 minutes (32%). A sample of the material eluting at 13.9 minutes was isolated and analyzed by negative ion electrospray mass spectrometry. The result showed strong ions at m/e 152 and 154, as expected for this compound, which has a molecular weight of 153 Daltons; the m/z 154 ion was due to the isotopic [^{14}C]atom. The radioactive peak eluting at 15.4 minutes was not isolated. However, in a separate HPLC experiment, it was shown to co-elute with synthetic N-acetyl-N-methyl-AMPA. N-methyl-[^{14}C]-AMPA has previously been shown to be an impurity in the initial [^{14}C]-AMPA material.

Under aseptic conditions, corn callus samples were transferred to individual wells of sterile 48-well COSTAR™ cell culture clusters (cat. No. 3548). The individual callus samples were not weighed. However, in several cases the total weight of the callus samples in a 48-well plate was determined. Typically, the average weight of individual callus samples was approximately 200–250 mg. In each assay, a nontransformed callus sample, HI II X B73, was included as a control. 50 µL of dose solution containing 3.3 X 10^6 dpm of [^{14}C]-glyphosate was added to each callus sample. 48-well plates were sealed with parafilm and placed in a plastic bag containing a wet paper towel to provide a moist atmosphere. Bags were closed and placed in a dark drawer at 25°C for 10 days. Each callus sample was subsequently transferred to a labeled microcentrifuge tube (VWR, 1.7-mL, cat. No. 20170-620). 1.0 mL of de-ionized water was added to each tube, and the tubes were closed and placed in round 20-tube floating microcentrifuge racks (Nalge cat. no. 5974-1015). These microfuge tubes were floated in boiling water for 30 minutes, shaken using a vortex mixer, and centrifuged for 5 minutes using a Fisher brand microcentrifuge. 120-µL supernatant samples were removed for analysis by HPLC as described below. The samples were injected using a Waters WISP autoinjector. Chromatographic profiles were obtained for each sample analyzed, and quantitative information was obtained by extrapolating the area under the radioactive elution peaks to total [^{14}C] in each sample. Figure 2 shows an HPLC profile of a mixture of standards of the observed radioactive metabolites [^{14}C] AMPA, [^{14}C] glyphosate, and N-acetyl-[^{14}C]-AMPA and the impurity identified as N-acetyl-N-methyl-[^{14}C]-AMPA.

HPLC analysis was typically completed using a SPHERISORB™ S5 SAX 250 mm x 10mm column for most analyses. Some samples were analyzed on an ALLTECH™ 5-micron, 250 x 10 mm SAX column, which provided similar performance. Two solvents were prepared. Solvent A consisted of 0.005 M KH_2PO_4, adjusted to pH 2.0 with H_3PO_4 and contained 4% methanol. Solvent B consisted of 0.10 M KH_2PO_4, adjusted to pH 2.0 with H_3PO_4 and also contained 4% methanol. The eluent flow rate was set at 3 mL/min, and the scintillation fluid flow rate was set at 9 mL/min using ATOMFLOW™ scintillation fluid (No. NEN-995, from Packard Instruments). All column solvent steps were linear, with the injection and column solvent flow rates as indicated in example 2. The column is prepared for an additional injection at 20 minutes.

Callus samples from 359 transformed corn lines were combined with 50-µL aliquots of [^{14}C]-glyphosate dose solution and incubated for 10 days in the dark. Each post-incubation callus sample, together with its clinging dose material, was transferred to a 1.7-mL microcentrifuge tube along with 1 mL of water, and each tube was placed in boiling water. This step causes cell lysis, releasing soluble intracellular compounds including any isotope labeled compounds such as glyphosate, AMPA, and N-acetyl-AMPA. It was determined during method development that if the post-incubation calli were rinsed thoroughly with water, 85–95% of the radioactivity was rinsed off, and HPLC analysis showed that virtually all of the radioactivity in the rinses was due to [^{14}C]-glyphosate and none was attributable to [^{14}C]-metabolites. In these experiments, the rinsed calli gave extracts containing [^{14}C]-metabolites in addition to [^{14}C]-glyphosate. This indicated that the radioactivity in the rinses was due mainly, if not exclusively, to unabsorbed surface [^{14}C]-glyphosate. It is important to take this into account when considering the rather low percentages of the dose converted to metabolites, because the percentage calculation includes large amounts of unabsorbed surface radioactivity. The method development work also showed that simply boiling the incubated calli in water released as much radioactivity as could be released by a conventional grinding/extracting procedure. Experiments were conducted to show that oiling did not alter the metabolite profiles. The streamlined procedures made it possible to analyze large numbers of samples (e.g., 96) at one time. Table 16 shows representative data of the callus samples producing the highest levels of N-acetyl-[^{14}C]-AMPA or [^{14}C]-AMPA obtained after HPLC analysis. A representative chromatogram of a

GOX plus AMPA acyltransferase transformed, glyphosate treated. callus extract sample is shown in Figure 3.

Table 16.

Transformed Corn Callus Lines Producing Amounts of AMPA or N-Acetyl-AMPA

Callus Producing N-Acetyl-[^{14}C]-AMPA			Callus Producing [^{14}C]-AMPA		
Callus *	Transformed with...	Percent ** N-Acetyl-[^{14}C]-AMPA	Callus *	Transformed with...	Percent ** [^{14}C]-AMPA
1978-05-02	pMON32570	0.27	1980-28-03	pMON32571	2.89
1978-08-01	pMON32570	0.94	OR523	pMON32926	2.00
1978-20-02	pMON32570	0.57	OR534	pMON32926	5.00
1978-21-02	pMON32570	0.23	OR537	pMON32926	2.00
1978-22-01	pMON32570	0.90	OR539	pMON32926	5.08
1978-24-02	pMON32570	1.80	1971-08-01	pMON32932	2.64
1978-35-01	pMON32570	0.22	1971-27-03	pMON32932	3.63
1980-01-01	pMON32570	0.27	OO505	pMON32932	2.73
1980-03-01	pMON32571	0.22	OO509	pMON32932	2.86
1981-28-01	pMON32571	0.25	OO510	pMON32932	2.34
1981-02-01	pMON32572	0.65	OO512	pMON32932	2.31
1981-03-01	pMON32572	0.74	OO514	pMON32932	1.98
1981-18-01	pMON32572	0.22	OO535	pMON32932	2.88
1981-23-01	pMON32572	0.48	OO538	pMON32932	2.70
1981-24-02	pMON32572	0.29	OO539	pMON32932	1.97
1981-32-02	pMON32572	1.08	OO553	pMON32932	3.56
1977-05-03	pMON32573	0.39	OO576	pMON32932	3.49
OR516	pMON32926	1.91	OO579	pMON32932	2.85
1972-14-01	pMON32931	0.40	1986-17-01	pMON32936	2.29
1972-32-01	pMON32931	0.75	1986-18-03	pMON32936	3.05
1972-33-01	pMON32931	0.55	1986-18-04	pMON32936	2.15
OO544	pMON32932	0.28	1986-28-02	pMON32936	2.06
1986-06-01	pMON32936	0.30	1983-12-02	pMON32938	2.41
1986-08-01	pMON32936	1.13	1983-31-01	pMON32938	2.90
1986-08-03	pMON32936	0.70	1985-03-02	pMON32946	2.51

Table 16. (continued)

Callus Producing N-Acetyl-[^{14}C]-AMPA			Callus Producing [^{14}C]-AMPA		
Callus *	Transformed with...	Percent ** N-Acetyl-[^{14}C]-AMPA	Callus *	Transformed with...	Percent ** [^{14}C]-AMPA
1986-12-01	pMON32936	0.33	1985-38-01	pMON32947	1.99
1986-18-02	pMON32936	0.40	OX512	pMON32948	2.43
1986-18-03	pMON32936	0.51	OX533	pMON32948	3.91
1986-18-04	pMON32936	1.09	OX556	pMON32948	12.11
1986-22-04	pMON32936	0.64	OY504	pMON32950	2.25
1983-11-01	pMON32938	0.21	OY511	pMON32950	2.53
OW534	pMON32946	0.77	OY528	pMON32950	2.58
OW542	pMON32946	0.85	OY532	pMON32950	2.24
1985-26-01	pMON32947	0.60	OY534	pMON32950	4.02
1985-26-03	pMON32947	0.71	OY535	pMON32950	2.34
1985-11-04	pMON32952	0.37	OY540	pMON32950	5.57

*All lines were transformed using ballistic methods. Lines designated by 19xx-yy-zz were transformed with isolated linear fragments of plasmids. Linear fragments were isolated so as to be separate from plasmid backbone structure.

** percent radioactivity detected for N-Acetyl-[^{14}C]-AMPA or [^{14}C]-AMPA peaks determined as a fraction of the total amount of radioactivity in the sample, including residual [^{14}C]-glyphosate as described in the text.

19 of the 359 callus samples tested produced extracts containing N-acetyl-[^{14}C]-AMPA at a level distinctly higher than the other callus samples. Callus OR516 was the strongest in this respect and was analyzed five times during a period of two months, providing values ranging from 0.50–4.54% (average 1.91%). The basis for the relatively large spread in the percentage of N-acetyl-[^{14}C]-AMPA formed at various times is unknown. In four of the five analyses of OR516, the percentage of N-acetyl-[^{14}C]-AMPA present was higher than that of [^{14}C]-AMPA, indicating an efficient conversion of [^{14}C]-AMPA to N-acetyl-[^{14}C]-AMPA. The callus next most efficient in producing N-acetyl-[^{14}C]-AMPA was 1978-24-02, which was the only other callus besides OR516 that contained more N-acetyl-[^{14}C]-AMPA than [^{14}C]-AMPA in its extract. One hundred of the 359 callus samples tested produced extracts containing [^{14}C]-AMPA at a level distinctly higher than other callus samples. OX556 was a superlative producer of [^{14}C]-AMPA, yielding more than twice as much of the metabolite as any other callus in the study. The control callus, HI II X B73, which contained no inserted genes, produced no detectable levels of N-acetyl-[^{14}C]-AMPA and only background levels of [^{14}C]-AMPA. This result indicates that

expression of an AMPA acyltransferase in corn is effective in conversion of AMPA produced as a result of GOX mediated glyphosate degradation to N-acetyl-AMPA.

Wheat

GOX mediated glyphosate degradation has been shown to produce AMPA, and AMPA has previously been shown to be the source of phytotoxic effects. Therefor, effects of wheat plant exposure to the compounds AMPA or N-acetyl-AMPA was determined as in example 2 in order to observe any wheat sensitivity or insensitivity to either of these compounds. The observation of any phytotoxic effects would indicate that GOX mediated glyphosate metabolism would be detrimental to *Triticum* species.

Wheat immature embryos were exposed to different concentrations of AMPA and N-acetyl-AMPA in a wheat embryo germination assay. MMSO base media was prepared containing 40 grams per liter maltose, 2 grams per liter GELRITE™, MS salts, and vitamins. Salts, vitamins, and maltose were dissolved in 3500 ml water and the pH was adjusted to 5.8. 500 ml was dispensed into a separate bottle along with 1 gram of GELRITE™ and autoclaved for 17 minutes. After the medium had cooled to about 45°C, AMPA or N-acetyl-AMPA was added to a defined concentration. The mixture was poured into six square Sundae cups under sterile conditions and allowed to solidify.

Immature wheat embryos were isolated from twenty day old seedlings (after anther formation) and inoculated into each MMSO media. Each Sundae cup contained nine immature embryo's. Three separate plates were used for each concentration of AMPA (0, 0.1, 0.15, 0.2, 0.25, 0.3, and 1.0 mM) or N-acetyl-AMPA (0, 0.1, 0.3, 1.0, and 3.0 mM). Sundae cups were incubated for ten days and the length of roots and shoots were determined and compared. The results are shown in Table 17.

Table 17.

Comparison of AMPA and N-acetyl AMPA on Germinating Shoot and Root Length

Phosphonate Compound AMPA (mM)	Shoot (cm)	Root (cm)
0.00	12.6 ± 2.6	7.0 ± 1.9
0.10	11.7 ± 2.5	8.0 ± 2.0
0.15	11.3 ± 2.1	6.3 ± 1.7
0.20	9.2 ± 1.8	4.6 ±2.1
0.25	8.5 ± 1.8	3.1 ± 1.6
0.30	6.6 ± 1.8	2.6 ± 1.6
1.00	0.9 ± 0.1	0.4 ± 0.1

N-Acetyl-AMPA		
0.00	12.6 ± 2.6	7.0 ± 1.9
0.10	12.0 ± 2.4	5.9 ± 1.4
0.30	11.7 ± 3.5	5.2 ± 1.2
1.00	12.2 ± 3.2	5.4 ± 1.5
3.00	11.2 ± 2.6	5.9 ± 1.6

AMPA was not substantially inhibitory to growth and elongation of immature embryo's at concentrations under 0.2 mM. However, concentrations above 0.2 mM were severely inhibitory to both shoot and root elongation, indicating that AMPA may also be phytotoxic to wheat and, considering the nature of the monocot crop species as a whole, phytotoxic to other monocotyledonous crops as well as turf grasses. Germination of immature embryo's was significantly affected when the AMPA level was higher than 0.20 mM. 1.00 mM AMPA eliminated the germination of immature embryo's in wheat. In contrast, N-acetyl-AMPA was not inhibitory to shoots and only mildly inhibitory to root elongation at any concentrations tested in this experiment. The highest N-acetyl-AMPA concentration tested was greater than ten times the minimal non-inhibitory concentration determined for AMPA. There are no significant effects to immature embryo germination when the N-acetyl-AMPA concentration is less than 3.0 mM. This result indicates that N-acetylation of AMPA in wheat would prevent AMPA phytotoxicity arising as a result of GOX mediated glyphosate herbicide metabolism.

Recombinant glyphosate tolerant wheat plants were generated according to the method of Zhou et al. (Plant Cell Reports 15:159 -163, 1995). Briefly, spring wheat, *Triticum aestivum* cv Bobwhite, was used as the target transformation line. Stock plants were grown in an environmentally controlled growth chamber with a 16 hour photoperiod at 800 microJoule per square meter per second provided by high-intensity discharge lights (Sylvania, GTE Products Corp., Manchester, NH). The day/night temperatures were 18/16°C. Immature caryopses were collected from the plants 14 days after anthesis. Immature embryos were dissected aseptically and cultured on MMS2 medium, a Murashige and Skoog (Physiol. Plant 15:473-497, 1962) basal medium supplemented with 40 grams per liter maltose and 2 milligrams per liter 2,4-D. In some experiments, CM4 medium was used. CM4 medium contains is MMS2 medium, but contains only 0.5 milligrams per liter 2,4-D and includes 2.2 milligrams per liter picloram. The immature embryos were cultured at 26°C in the dark.

Immature embryos were transferred five days after culture initiation to an osmotic treatment CM4 medium containing 0.35 M mannitol four hours prior to bombardment according to the method of Russell et al. (In Vitro Cell Devel. Biol., 28P:97-105, 1992). Thirty to forty embryos were placed in the center of each plate and bombarded in a DuPont PDS1000 apparatus. Plasmid DNA was adsorbed onto 1 µm tungsten particles according to the method of Sanford et al. (Particle Sci. Technol., 5:27-37, 1987). Embryos were bombarded twice at a distance of 13 mm from the stopping plate. A 100 µm stainless steel screen was placed immediately below the stopping plate.

After a 16 hour post bombardment treatment on the osmotic medium, the embryos were transferred to MMS2 or CM4 medium. Following a one week delay, the embryos were transferred to the MMS2 or CM4 medium containing 2 mM glyphosate. After 9-12 weeks of callus proliferation on the selection medium, calli were transferred to a MMS0.2 regeneration medium containing 0.2 mg/l 2,4-D and 0.1 mM glyphosate. Shoots obtained from the regeneration medium were transferred to MMS0 without 2,4-D but containing 0.02 mM glyphosate.

Glyphosate tolerant R_0 plants as well as R_1 progeny were transferred to 15 centimeter diameter pots and grown in an environmentally controlled chamber as described above. Two weeks later, the plants were sprayed with 3 ml/liter ROUNDUP (41% active ingredient, Monsanto Company) in a spray chamber, which was designed to mimic a field dose application of 0.6 kilograms glyphosate per hectare. Damage symptoms were observed and recorded at different stages following the spraying.

Genomic DNA was isolated from leaf tissue of R_0 and R_1 progeny following the method of Shure et al. (Cell 35:225-233, 1983). Fifteen micrograms of genomic DNA was digested with *Bgl*II restriction endonuclease and fractionated on a 0.8% agarose gel. The DNA was transferred to Hybond N membranes (Amersham) according to the standard procedure described in Sambrook et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989). The membranes were probed independently for the presence of genes encoding EPSPS and GOX. A 3.4 kb DNA fragment containing the EPSPS gene and a 4.8 kb DNA fragment containing the GOX gene were released from pMON19574 by *Bgl*II restriction endonuclease digestion, isolated by 0.7% agarose gel electrophoresis and labeled with [^{32}P] dCTP using a Stratagene PRIME-IT II random primer labeling kit. Probes were labeled to a

specific activity of 3 X 10^9 counts per minute per microgram and 1.3 X 10^9 counts per minute per microgram, respectively. Membranes were hybridized for 14 hours at 42°C in a solution containing 50% formamide, 5X SSC, 5X Denhardt's, 0.5% SDS, and 100 microgram per milliliter tRNA. The condition of the final wash was 0.1% SSC and 0.1% SDS at 60°C for fifteen minutes.

EPSPS and GOX protein assays were conducted using crude protein extracts from leaf tissue of R_0 plants and total proteins were quantified following the method of Bradford (Anal. Biochem. 72:248-256, 1976). The percentage of EPSPS and GOX protein represented in the extracts was quantified using an ELISA method and calculated as percent total extractable protein.

Immature embryos from the R_0 transgenic and Bobwhite control plants were isolated twenty days after anthesis and cultured on the MMS0 medium with 0.02 mM glyphosate for a germination test. Germinated and non-germinated embryos were recorded ten days later and the data was analyzed by χ^2 test for 3:1 segregation. Tolerant plants from the germination test were transferred to soil and sprayed with three milliliters per liter of ROUNDUP as described above.

Five plasmids harboring glyphosate resistance genes were used to transform immature wheat embryos as described above. pMON19338 contains a nucleotide cassette encoding a petunia EPSPS chloroplast transit peptide in frame with an *Agrobacterium* strain CP4 EPSPS enzyme sequence. The nucleotide cassette is inserted downstream of a cauliflower mosaic virus enhanced 35S promoter linked 3' to a maize HPS70 intron sequence and upstream of a nopaline synthase 3' transcription termination and polyadenylation sequence. Convenient restriction sites are positioned between the intron sequence and the 3' termination sequence for insertion of genetic elements. pMON19643 is identical to pMON19338 except that a GOX enzyme encoding sequence is used in place of the *Agrobacterium* EPSPS enzyme encoding sequence. pMON19574 is identical to pMON19338 but additionally contains a chloroplast targeted glyphosate-oxidoreductase expression cassette identical to that in pMON19643 downstream of and immediately adjacent to the EPSPS expression cassette. pMON32570 is similar to pMON19574 in that expression cassettes encoding a chloroplast targeted EPSPS and chloroplast targeted GOX are present, however, an expression cassette encoding a chloroplast targeted AMPA acyltransferase enzyme is also present between the EPSPS and GOX expression cassettes. Other elements which are present in pMON19574 and not in the other plasmids are

also worthy of mention. For example, a wheat major chlorophyll a/b binding protein gene 5' untranslated leader is present between the enhanced 35S promoter and intron in both the EPSPS and AMPA acyltransferase expression cassettes (McElroy et al., Plant Cell 2:163-171, 1990). Also, a wheat hsp17 gene 3' transcription termination and polyadenylation sequence is present in place of the nopaline synthase 3' sequence for both EPSPS and AMPA acyltransferase expression cassettes. All plasmids produced recombinant glyphosate tolerant wheat plants using the ballistic transformation method described above. However, plasmids which were capable of expressing GOX only or GOX along with an AMPA acyltransferase either did not produce recombinant glyphosate tolerant wheat plants or produced plants which experienced problems with stunted growth, aberrant segregation of phenotypes, and infertility and were not analyzed further. The data obtained after biolistic transformation using the described plasmids is shown in Table 18.

Table 18. Wheat Biolistic Transformation Data

Glyphosate Tolerance Gene(s)	# Explants	# Transgenic Events	Transformation Efficiency[1]
GOX	120	0	0
GOX + PhnO	434	6	1.4
EPSPS	120	6	5.0
EPSPS + GOX	120	1	0.8
EPSPS + PhnO + GOX	10,068	314	3.1

1 - transformation efficiency based on percentage of transgenic events identified from a total population of explants arising from a combination of experiments in which a particular vector construct has been bombarded into immature embryo's.

Transformed glyphosate tolerant plants arising out of these transformations were self crossed and allowed to produce R1 seed, which were used to generate R1 plants. Glyphosate tolerance generally segregated in the expected ratio of 3:1 in R1 plants as judged by R1 plant sensitivities after spraying with glyphosate at the three leaf stage. Glyphosate tolerant R1 plants were self crossed and allowed to produce R2 seed. R2 seed was germinated from a number of different glyphosate tolerant lines to produce R2 glyphosate tolerant plants to which [^{14}C]-glyphosate was applied as described above. Plant leaf and stem tissues were harvested at 48 hours after glyphosate application, and water soluble compounds were extracted as described above and analyzed by HPLC as in example 2 for the presence of [^{14}C]-glyphosate metabolites. The total area under the [^{14}C] isotope labeled peaks eluting from the column was summed to

provide a baseline of 100% [^{14}C]-compound identification for each sample analyzed. The results are shown in Table 19.

Table 19. Glyphosate Metabolism In Wheat Plant Extracts[1]

Sample & Glyphosate Tolerance Gene(s)	Plant Line No.	[^{14}C]-Glyphosate	[^{14}C]-AMPA	Acetyl-[^{14}C]-AMPA	[^{14}C]-Other[4]
Standard[2]	na	30	26	31	13
	na	29	24	29	18
	na	35	29	36	0
Growth Medium[3]	na	60	32	0.2	8
	na	48	25	2	25
	na	87	7	0	6
EPSPS	24756	43	25	1	31
	24756	53	46	0	1
	25397	61	38	0	1
	25397	37	19	1.2	43
	25397	64	20	0	16
EPSPS + PhnO + GOX	27249	6	7	85	2
	27249	14	12	61	13
	27249	5	24	33	38
	25462	48	21	0	31
	25462	44	5	0	51
	25462	54	35	0	11
	26281	48	14	17	21
	26281	64	11	13	12
	26281	38	7	7	48
	28598	20	7	5	68
	28598	25	7	5	63
Bobwhite	na	74	26	0	0
	na	17	15	0	32
	na	34	24	0	42

1 - plant tissue extracts were analyzed by HPLC after [^{14}C]-glyphosate application as in Example 1, and the area under the plots for each peak were summed to provide a base of 100% [^{14}C]-compound identification for each sample.
2 - standard solution containing approximately equal [^{14}C] molar ratios of each known glyphosate metabolism related compound.
3 - growth medium including [^{14}C]-glyphosate; glyphosate has previously been shown to be degraded by a photolytic process to AMPA, which can be autoacylated in the presence of certain acyl compounds (MSL-0598).
4 - uncharacterized [^{14}C]-labeled compounds which are resolved using the disclosed chromatographic method. Retention time of glyphosate is about 9.6 minutes, AMPA is about 5.4 minutes, N-acetyl-AMPA is about 12.5 minutes, and the major [^{14}C]-labeled impurity in the [^{14}C]-glyphosate sample is about 4.7 minutes.

The standard solution contains approximately equal molar ratios of each of the compounds glyphosate, AMPA and N-acetyl-AMPA, as well as a number of impurities which are present as a result of the chemical synthesis of these isotope labeled compounds. Growth medium to which [^{14}C]-glyphosate was added was treated to the same conditions as wheat plants, ie, the medium was exposed to incident light intensities which plants received. As expected, photodegradation of glyphosate to AMPA was observed, and a small percentage of AMPA appeared to be converted to acetyl-AMPA, probably as a result of exposure in the growth medium to other acylated compounds. Photodegradation of glyphosate by visible light exposure to AMPA as the major degradation product has been observed previously (Lund-Hoeie et al., *Photodegradation of the herbicide glyphosate in water*. Bull. Environ. Contam. Toxicol. 36:723-729, 1986). Recombinant wheat plants transformed with an EPSPS-only plasmid did not produce [^{14}C]-AMPA or acetyl-[^{14}C]-AMPA from [^{14}C]-glyphosate. [^{14}C]-AMPA and trace amounts of acetyl-[^{14}C]-AMPA which were observed were within the limits observed as a result of photodegradation in the growth medium control. Non-recombinant Bobwhite control plants treated with [^{14}C]-glyphosate also did not produce AMPA or acetyl-AMPA. Plants transformed with the triple gene construct plasmid containing genes capable of expressing EPSPS, PhnO and GOX produced variable results. About one third of these plants appeared to efficiently convert glyphosate to acetyl-AMPA, indicating that the GOX and PhnO enzymes were present and functional. Southern blot analyses demonstrated that the transgenes were integrated into the wheat genomes and transmitted to the following generations. Western blot analysis using anti-EPSPS, anti-GOX, or anti-PhnO antiserum to detect these proteins in the triple gene transformed plant extracts provided further insight into the basis for the variable [^{14}C]-glyphosate metabolism observation. Western blot analysis indicated that all of the lines were producing EPSPS, however only line 27249 was producing GOX and PhnO protein. This result is consistent with the data in Table 19, which shows that line 27249 efficiently metabolizes [^{14}C]-glyphosate to acetyl-[^{14}C]-AMPA. This plant line also did not demonstrate stunting, partial fertility, or altered segregation phenotypes associated with other lines. These results indicate that co-expression of GOX and AMPA acyltransferase in wheat plants expressing recombinant EPSPS provides improved herbicide tolerance.

EXAMPLE 9

This example illustrates the transformation of tobacco chloroplasts with a *phn*O gene.

Recombinant plants can be produced in which only the mitochondrial or chloroplast DNA has been altered to incorporate the molecules envisioned in this application. Promoters which function in chloroplasts have been known in the art (Hanley-Bowden et al., Trends in Biochemical Sciences **12**:67-70, 1987). Methods and compositions for obtaining cells containing chloroplasts into which heterologous DNA has been inserted have been described, for example by Daniell et al. (U.S. Pat. No. 5,693,507; 1997) and Maliga et al. (U.S. Pat. No. 5,451,513; 1995). A vector can be constructed which contains an expression cassette from which an acyltransferase protein could be produced. A cassette could contain a chloroplast operable promoter sequence driving expression of, for example, a *phn*O gene, constructed in much the same manner as other polynucleotides herein, using PCR methodologies, restriction endonuclease digestion, and ligation etc. A chloroplast expressible gene would provide a promoter and a 5' untranslated region from a heterologous gene or chloroplast gene such as *psb*A, which would provide for transcription and translation of a DNA sequence encoding an acyltransferase protein in the chloroplast; a DNA sequence encoding an acyltransferase protein; and a transcriptional and translational termination region such as a 3' inverted repeat region of a chloroplast gene that could stabilize an expressed mRNA coding for an acyltransferase protein. Expression from within the chloroplast would enhance gene product accumulation. A host cell containing chloroplasts or plastids can be transformed with the expression cassette and then the resulting cell containing the transformed chloroplasts can be grown to express the acyltransferase protein. A cassette may also include an antibiotic, herbicide tolerance, or other selectable marker gene in addition to the acyltransferase gene. The expression cassette may be flanked by DNA sequences obtained from a chloroplast DNA which would facilitate stable integration of the expression cassette into the chloroplast genome, particularly by homologous recombination. Alternatively, the expression cassette may not integrate, but by including an origin of replication obtained from a chloroplast DNA, would be capable of providing for replication of, for example, a heterologous *phn*O or other acyltransferase gene within the chloroplast.

Plants can be generated from cells containing transformed chloroplasts and can then be grown to produce seeds, from which additional plants can be generated. Such transformation methods are advantageous over nuclear genome transformation, in particular where chloroplast

transformation is effected by integration into the chloroplast genome, because chloroplast genes in general are maternally inherited. This provides environmentally "safer" transgenic plants, virtually eliminating the possibility of escapes into the environment. Furthermore, chloroplasts can be transformed multiple times to produce functional chloroplast genomes which express multiple desired recombinant proteins, whereas nuclear genomic transformation has been shown to be rather limited when multiple genes are desired. Segregational events are thus avoided using chloroplast or plastid transformation. Unlike plant nuclear genome expression, expression in chloroplasts or plastids can be initiated from only one promoter and continue through a polycistronic region to produce multiple peptides from a single mRNA.

The expression cassette would be produced in much the same way that other plant transformation vectors are constructed. Plant chloroplast operable DNA sequences can be inserted into a bacterial plasmid and linked to DNA sequences expressing desired gene products, such as PhnO proteins or other similar acyltransferases, so that the acyltransferase protein is produced within the chloroplast, obviating the requirement for nuclear gene regulation, capping, splicing, or polyadenylation of nuclear regulated genes, or chloroplast or plastid targeting sequences. An expression cassette comprising a *phn*O or similar acyltransferase gene, which is either synthetically constructed or a native gene derived directly from an *E. coli* genome, would be inserted into a restriction site in a vector constructed for the purpose of chloroplast or plastid transformation. The cassette would be flanked upstream by a chloroplast or plastid functional promoter and downstream by a chloroplast or plastid functional transcription and translation termination sequence. The resulting cassette could be incorporated into the chloroplast or plastid genome using well known homologous recombination methods.

Alternatively, chloroplast or plastid transformation could be obtained by using an autonomously replicating plasmid or other vector capable of propagation within the chloroplast or plastid. One means of effectuating this method would be to utilize a portion of the chloroplast or plastid genome required for chloroplast or plastid replication initiation as a means for maintaining the plasmid or vector in the transformed chloroplast or plastid. A sequence enabling stable replication of a chloroplast or plastid epigenetic element could easily be identified from random cloning of a chloroplast or plastid genome into a standard bacterial vector which also contains a chloroplast or plastid selectable marker gene, followed by transformation of chloroplasts or plastids and selection for transformed cells on an appropriate selection medium.

Introduction of an expression cassette as described herein into a chloroplast or plastid replicable epigenetic element would provide an effective means for localizing an acyltransferase gene and protein to the chloroplast or plastid.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantageous results attained. As various changes could be made in the above methods and compositions without departing from the spirit and scope of the invention, it is intended that all matter contained in the above description, and shown in the accompanying drawings and sequences, shall be interpreted as illustrative and not in a limiting sense.

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McBride et al., WO 95/24492.

Claims:

1. A recombinant plant transformed with a polynucleotide sequence comprising:
   a) a plant functional promoter sequence operably linked to;
   b) a structural DNA sequence which encodes an acyltransferase enzyme, operably linked to;
   c) a 3' sequence which functions in plants to cause transcription termination;
   wherein the promoter sequence causes sufficient expression of said enzyme in plant tissue to enhance the phosphonate herbicide tolerance of a plant transformed with said polynucleotide sequence, and wherein said enzyme transfers an acyl group from an acylated donor compound to the terminal amine of a phosphonate herbicide.
2. The plant of claim 1 in which said acyltransferase enzyme is localized to plastids in said plant.
3. The plant of claim 2 wherein said plastids comprise chloroplasts.
4. The plant of claim 3 in which said structural DNA sequence comprises a 5' sequence encoding an amino-terminal chloroplast transit peptide operably linked 5' to said structural DNA sequence, wherein said enzyme is localized to the plant chloroplasts or plastids.
5. The plant of claim 2, wherein said acylated donor is an acyl coenzyme A.
6. The plant of claim 5 wherein said acyl coenzyme A is selected from the group consisting of acetyl coenzyme A, propionyl coenzyme A, malonyl coenzyme A, succinyl coenzyme A, and methyl-malonyl coenzyme A.
7. The plant of claim 6 wherein said acyl coenzyme A is acetyl coenzyme A.
8. The plant of claim 6 selected from the group consisting of corn, wheat, cotton, rice, soybean, sugarbeet, canola, flax, barley, oilseed rape, sunflower, potato, tobacco, tomato, alfalfa, lettuce, apple, poplar, pine, eucalyptus, acacia, poplar, sweetgum, radiata pine, loblolly pine, spruce, teak, alfalfa, clovers and other forage crops, turf grasses, oilpalm, sugarcane, banana, coffee, tea, cacao, apples, walnuts, almonds, grapes, peanuts, pulses, petunia, marigolds, vinca, begonias, geraniums, pansy, impatiens, oats, sorghum, and millet.

9. The plant of claim 2 in which the promoter sequence is derived from a plant DNA virus promoter sequence.

10. The plant of claim 9 in which said promoter sequence is selected from the group consisting of CaMV35S, FMV35S, enhanced CaMV35S, enhanced FMV35S, comalina yellow mottle virus promoter, and sugar cane bacilliform DNA virus promoter.

11. The plant of claim 10 in which the structural DNA sequence is or is complementary to an *E. coli phnO* gene sequence as set forth in SEQ ID NO:3.

12. The plant of claim 11 wherein said structural DNA sequence encodes a peptide selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:8.

13. The plant of claim 12 in which the structural DNA sequence is derived from a microbe, wherein said sequence is or is complementary to a polynucleotide sequence capable of hybridizing to a sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:11, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, and SEQ ID NO:19.

14. The plant of claim 13 in which the acyltransferase enzyme is substantially similar to an *E. coli* PhnO peptide which functions in plants to transfer an acyl group from an acylated donor compound to the terminal amine of a phosphonate herbicide.

15. The plant of claim 14 wherein said phosphonate herbicide is selected from the group consisting of glyphosate and AMPA.

16. The plant of claim 4 in which the 5' sequence encoding an amino-terminal chloroplast transit peptide is selected from the group consisting of SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, and SEQ ID NO:14.

17. The plant according to claim 3 which exhibits enhanced tolerance to one or more phosphonate herbicides, the herbicide being selected from the group consisting of glyphosate and AMPA.

18. A seed produced from the plant of claim 17, wherein said seed comprises said polynucleotide sequence.

19. A plant grown from the seed of claim 18.

20. A stably transformed phosphonate herbicide tolerant recombinant plant which contains a polynucleotide sequence comprising:

a) a plant functional promoter sequence operably linked to;

b) a structural DNA sequence which encodes an acyltransferase enzyme, operably linked to;

c) a 3' sequence which functions in plants to cause transcription termination;

wherein the promoter sequence is heterologous with respect to the structural DNA sequence and causes sufficient expression of said enzyme in plant tissue to enhance the phosphonate herbicide tolerance of a plant transformed with said polynucleotide sequence, wherein said enzyme transfers an acyl group from an acylated donor to the terminal amine of a phosphonate herbicide, and wherein said plant expresses a GOX gene which encodes a plant functional glyphosate oxidoreductase enzyme.

21. A method for selectively enhancing phosphonate herbicide tolerance in a recombinant plant comprising the steps of:

a) transforming said plant with a polynucleotide sequence comprising

i) a promoter sequence which functions in plants to cause the production of an RNA sequence, operably linked to;

ii) a structural DNA sequence capable of producing an RNA sequence which encodes an acyltransferase enzyme, operably linked to;

iii) a 3' non-translated sequence which functions in plants to cause the addition of a polyadenylated nucleotide sequence to the 3' end of said RNA sequence;

wherein the promoter sequence is heterologous with respect to the structural DNA sequence and causes sufficient expression of said enzyme in plant tissue to enhance the phosphonate herbicide tolerance of a plant transformed with said polynucleotide sequence, wherein said enzyme transfers an acyl group from an acylated donor substrate to the terminal amine of a phosphonate herbicide substrate, and wherein said plant expresses a GOX gene which encodes a plant functional glyphosate oxidoreductase enzyme; and

b) expressing a herbicide tolerant effective amount of said acyltransferase enzyme in said plant.

22. The method according to claim 21, wherein said acyltransferase is expressed from a DNA sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:11, and SEQ ID NO:19.

23. A method for producing a genetically transformed phosphonate herbicide tolerant plant comprising the steps of:

a) inserting into the genome of a plant cell a polynucleotide sequence comprising;

i) a promoter sequence which functions in plant cells to cause the production of an RNA sequence, operably linked to;

ii) a structural DNA sequence capable of producing an RNA sequence which encodes an acyltransferase enzyme which transfers an acyl group from an acylated donor to the terminal amine of a phosphonate herbicide substrate, operably linked to;

iii) a 3' non-translated sequence which functions in plant cells to cause the addition of a polyadenylated nucleotide sequence to the 3' end of said RNA sequence;

wherein the promoter sequence is heterologous with respect to the structural DNA sequence and causes sufficient expression of said enzyme in a plant cell to enhance the phosphonate herbicide tolerance of a plant cell transformed with said polynucleotide sequence;

b) selecting a transformed plant cell; and

c) regenerating from the transformed plant cell a genetically transformed plant which exhibits improved phosphonate herbicide tolerance.

24. A method for producing a genetically transformed phosphonate herbicide tolerant plant comprising the steps of:

a) inserting into the genome of a plant cell a polynucleotide sequence comprising:

i) a promoter sequence which functions in plant cells to cause the production of an RNA sequence, operably linked to;

ii) a structural DNA sequence capable of producing an RNA sequence which encodes an acyltransferase enzyme which transfers an acyl group from an acylated donor to the terminal amine of a phospho..ate herbicide substrate, operably linked to;

iii) a 3' non-translated sequence which functions in plant cells to cause the addition of a polyadenylated nucleotide sequence to the 3' end of said RNA sequence;

wherein the promoter sequence is heterologous with respect to the structural DNA sequence and causes sufficient expression of said enzyme in a plant cell to enhance the phosphonate herbicide tolerance of a plant cell transformed with said polynucleotide sequence, and wherein said plant cell expresses a GOX gene which encodes a glyphosate oxidoreductase enzyme;

b) selecting a transformed plant cell; and

c) regenerating from the transformed plant cell a genetically transformed plant which exhibits improved phosphonate herbicide tolerance.

25. The method according to claim 24, wherein a transformed plant cell is selected by an ability to grow in the presence of a selective agent, wherein said agent is selected from the group consisting of glyphosate and AMPA.

26. A peptide comprising an acyltransferase enzyme that catalyzes the transfer of an acyl group from an acylated donor to the terminal amine of a phosphonate herbicide.

27. The peptide according to claim 26 wherein the acyltransferase enzyme is comprised of a sequence selected from the group consisting of SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:12, and SEQ ID NO:20.

28. The peptide according to claim 26 wherein said phosphonate herbicide is selected from the group consisting of glyphosate and AMPA.

29. The peptide according to claim 26 wherein the acyltransferase enzyme is expressed in a cell from a DNA sequence which is or hybridizes to a polynucleotide sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:7, SEQ ID NO:11, and SEQ ID NO:19.

30. The peptide according to claim 27 wherein the DNA sequence is derived from a microbe, said microbe being a member of the genus selected from the group consisting of Enterobacteriaceae, Streptomyces, Bacillus, Actinobacillus, Ascomycota, and Basidiomycota.

31. A method for selecting one or more cells transformed with a vector containing an acyltransferase gene encoding an enzyme which functions to N-acetylate a phosphonate herbicide compound comprising the steps of:

a) transforming a population of cells with said vector;

b) incubating said transformed cells in the presence of an inhibitory amount of phosphonate herbicide compound;

c) identifying one or more cells that grow in the presence of said inhibitory amount of said compound; and

d) isolating and purifying said one or more cells that grow in the presence of said inhibitory amount of said compound.

32. A vector according to claim 31 comprising an acyltransferase gene encoding an enzyme which functions in said one or more cells to transfer an acyl group from an acylated donor substrate to the terminal amine of a phosphonate herbicide compound.

33. The vector according to claim 31 wherein the gene is expressed in a host cell.

34. The vector according to claim 33 wherein the host cell is not inhibited by the presence of a phosphonate herbicide in an amount which is inhibitory to a host cell lacking a functional acyltransferase gene encoding said enzyme.

35. The host cell according to claim 34 selected from the group consisting of bacterial cells, fungal cells, animal cells, and plant cells.

36. The host cell according to claim 35 wherein the bacterial cell is selected from the bacterial species consisting of Enterobacteriaceae, Mycobacteriaceae, Agrobacteriaceae, Actinobacteriaceae, Streptomyces, and Bacillus.

37. The host cell according to claim 35 wherein the fungal cell is selected from the fungal species consisting of Ascomycota, Basidiomycota, and Deuteromycota.

38. The host cell according to claim 35 wherein the plant cell is selected from the plant species consisting of Glycine max, Zea mays, Nicotania tabacum, Gossypium gossypia, Triticum aestivum, and Brassica napus.

39. An antibody which binds to an acyltransferase protein sequence, wherein said acyltransferase is selected from the group consisting of SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:12, and SEQ ID NO:20.

40. A method for identifying a recombinant acyltransferase gene in a sample comprising

a) providing one or more distinct polynucleotide sequences capable of hybridizing to said gene;

b) providing a reference sample comprising one or more polynucleotide sequences complementary to said distinct polynucleotide sequences; and

c) providing instructions for combining said distinct sequences, said reference sample, and said recombinant acyltransferase gene in a sample, and

d) detecting the recombinant gene in said sample.

41. A kit for detecting the presence of a recombinant acyltransferase gene in a sample comprising

a) providing one or more distinct polynucleotide sequences capable of hybridizing to said gene;

b) providing a reference sample comprising one or more polynucleotide sequences complementary to said distinct polynucleotide sequences; and

c) providing instructions for combining said distinct sequences, said reference sample, and said recombinant acyltransferase gene in a sample

packaged together in a kit.

42. A plant comprising a polynucleotide sequence containing a gene which encodes an acetyltransferase protein, wherein expression of said gene in said plant stimulates said plant's growth.

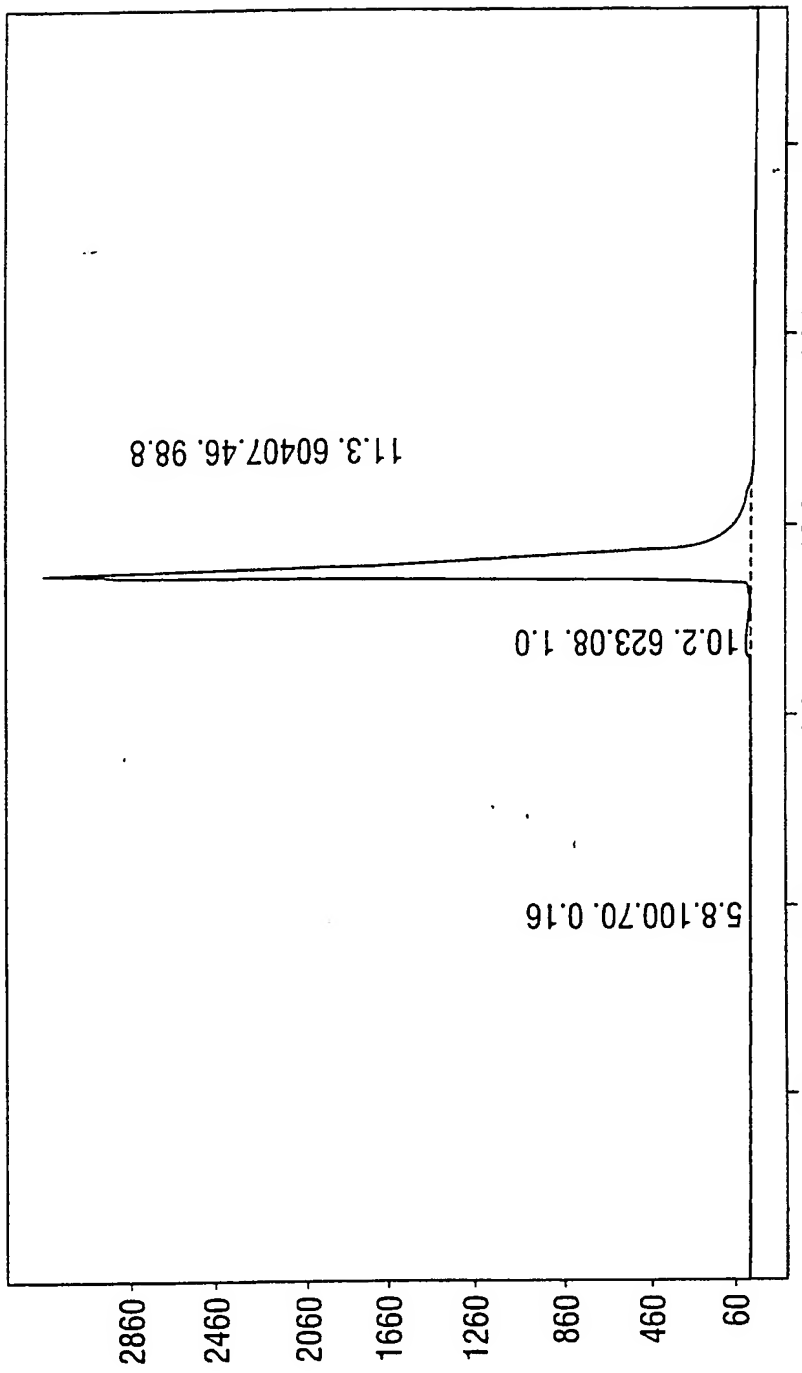
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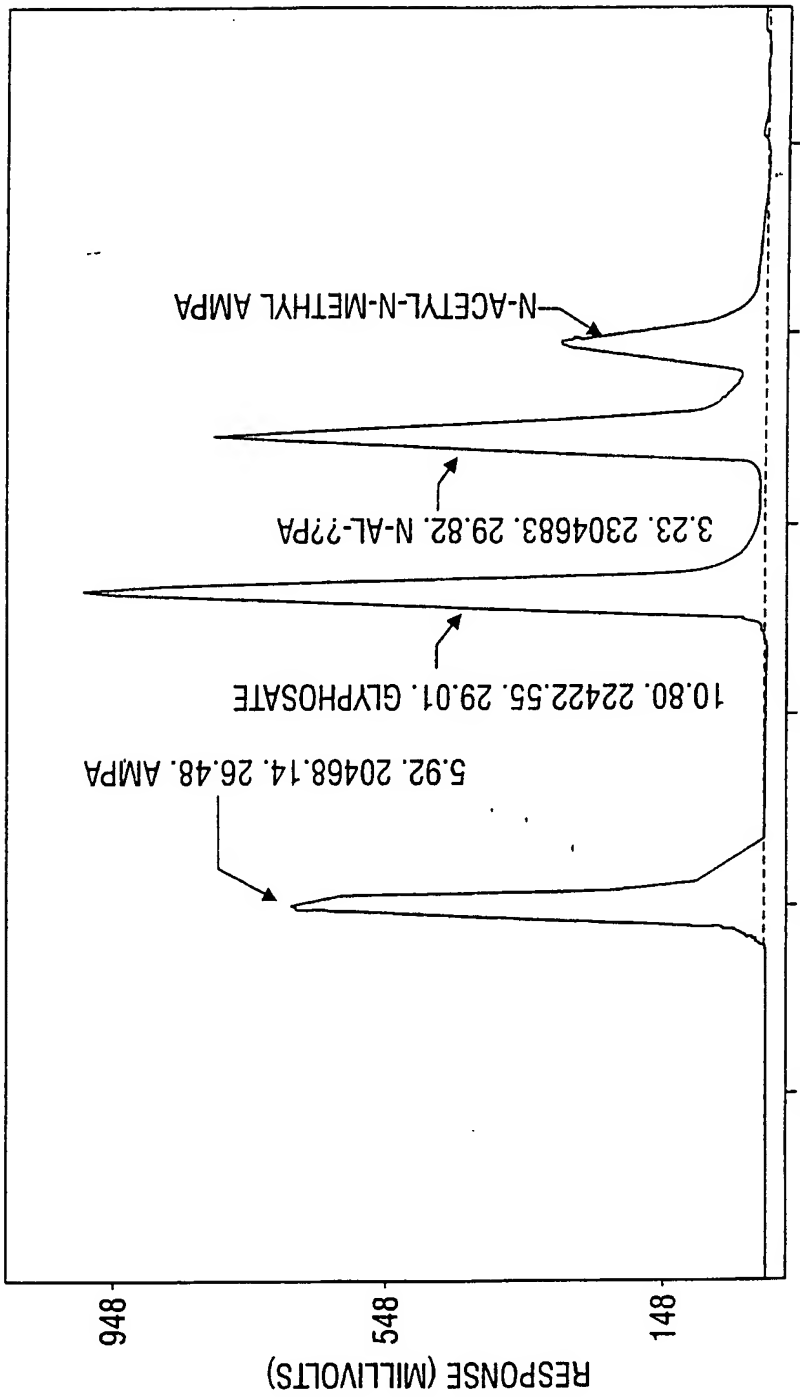
FIG. 1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9.0
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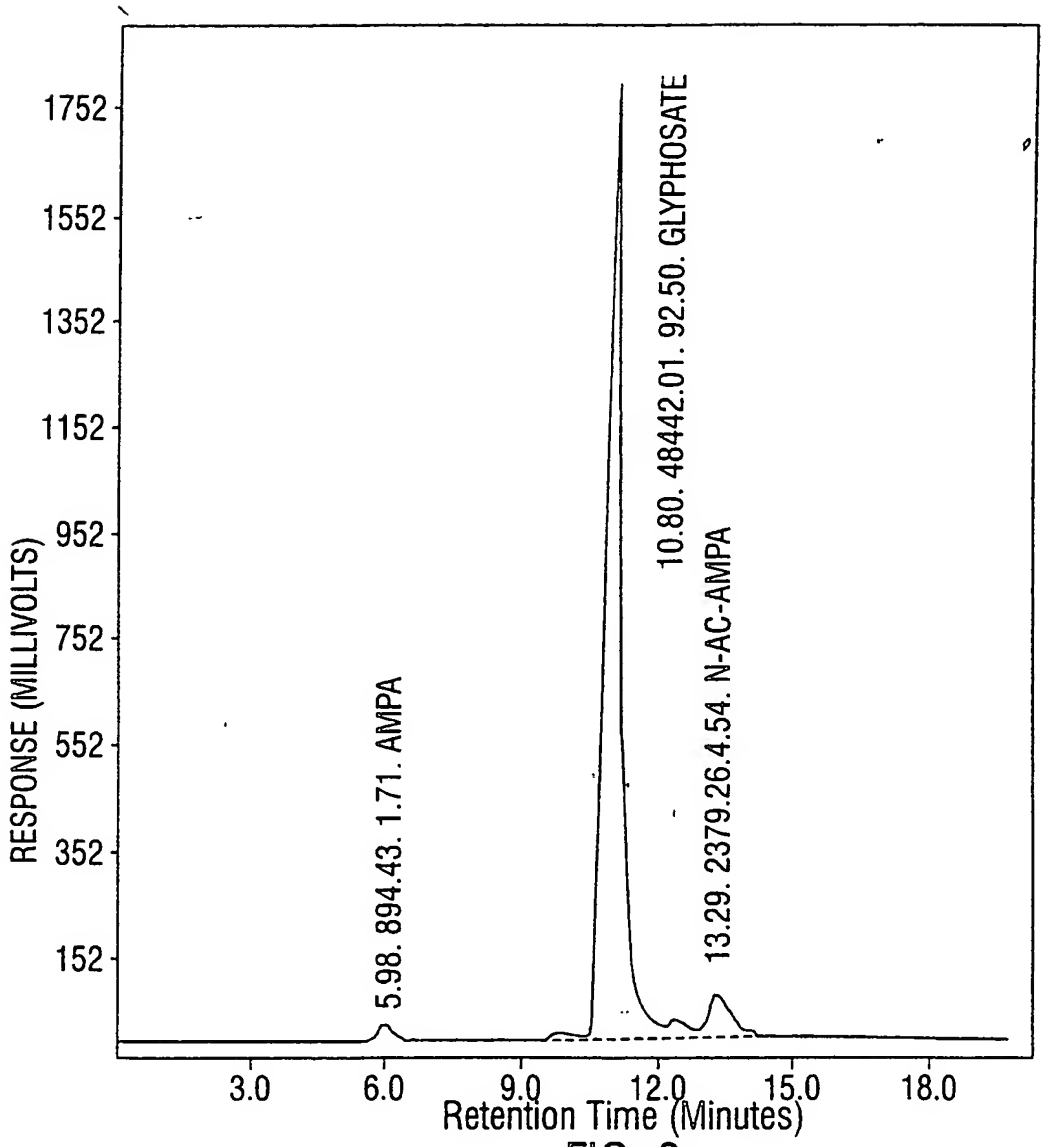
FIG. 2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12.0
15.0
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Retention Time (Minutes)

FIG. 3

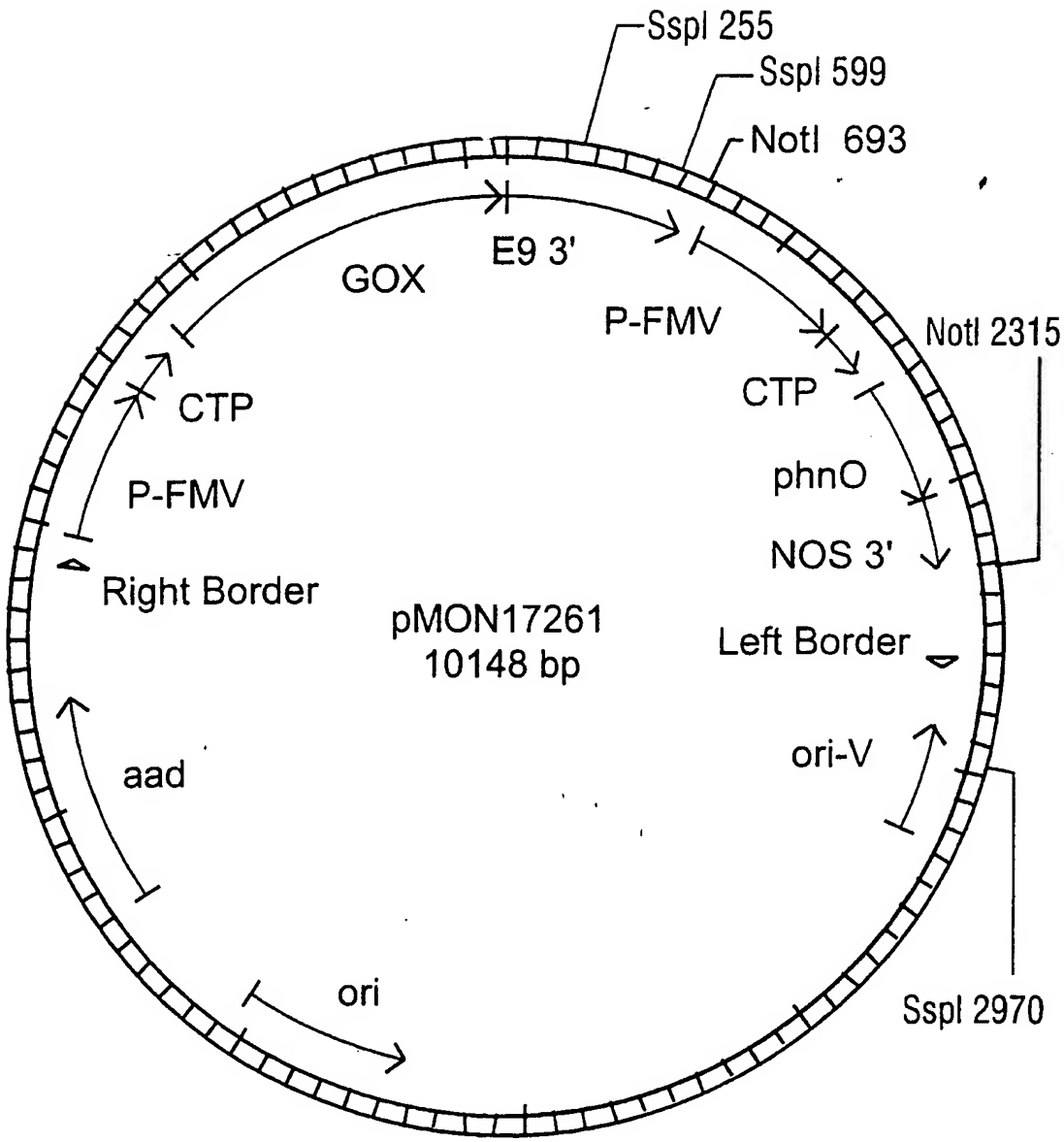
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FIG. 4

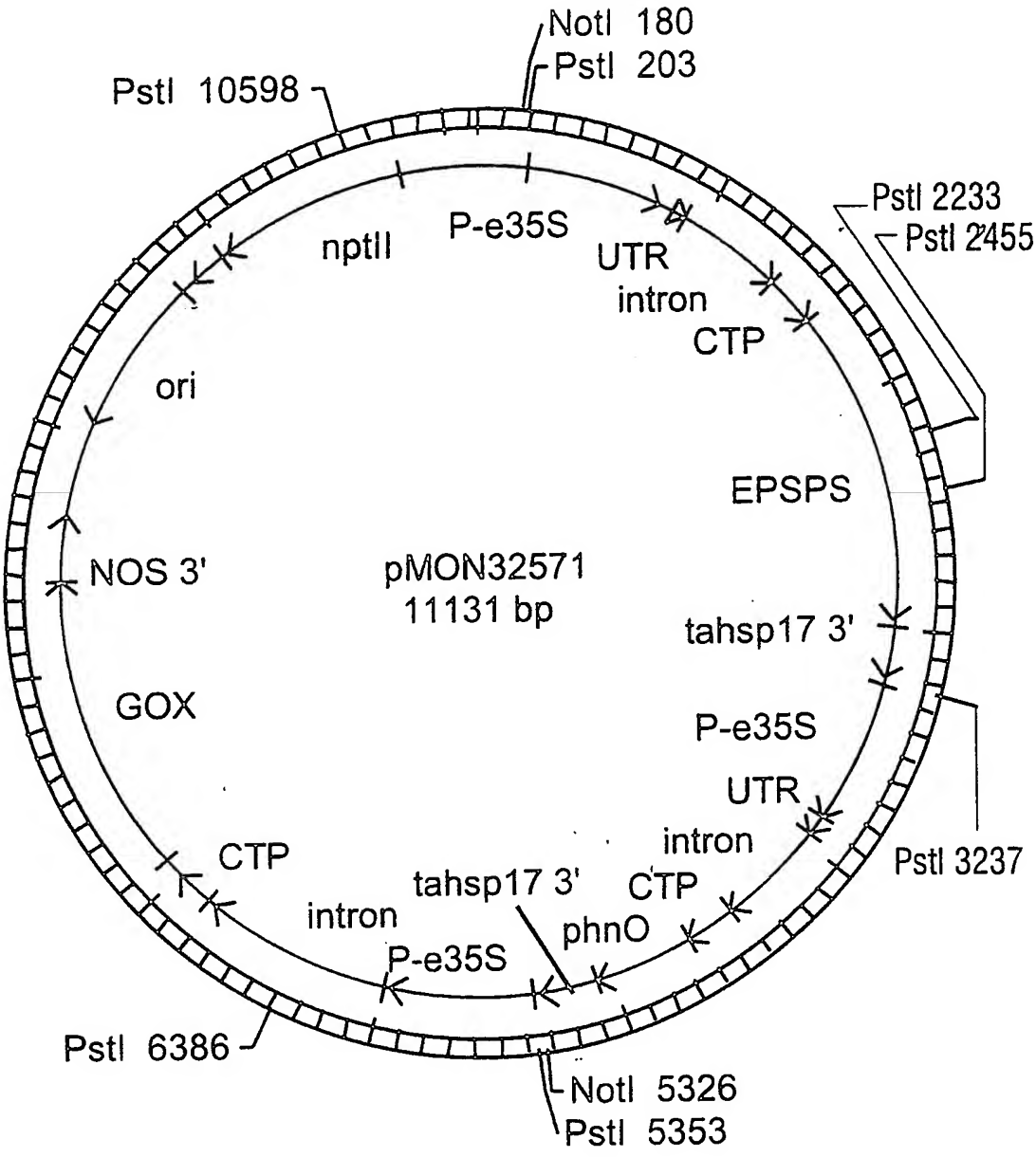
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FIG. 5

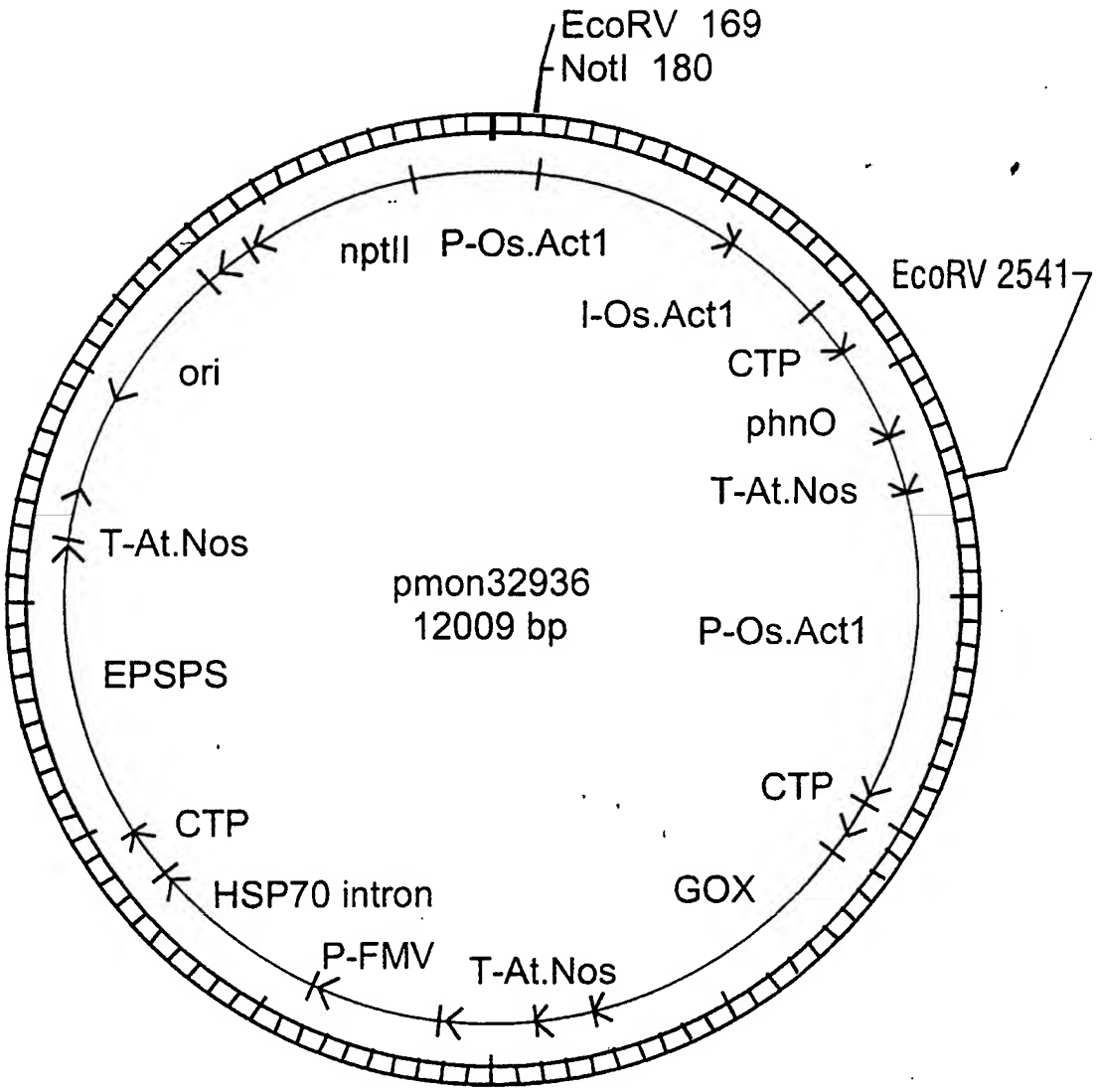
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FIG. 6

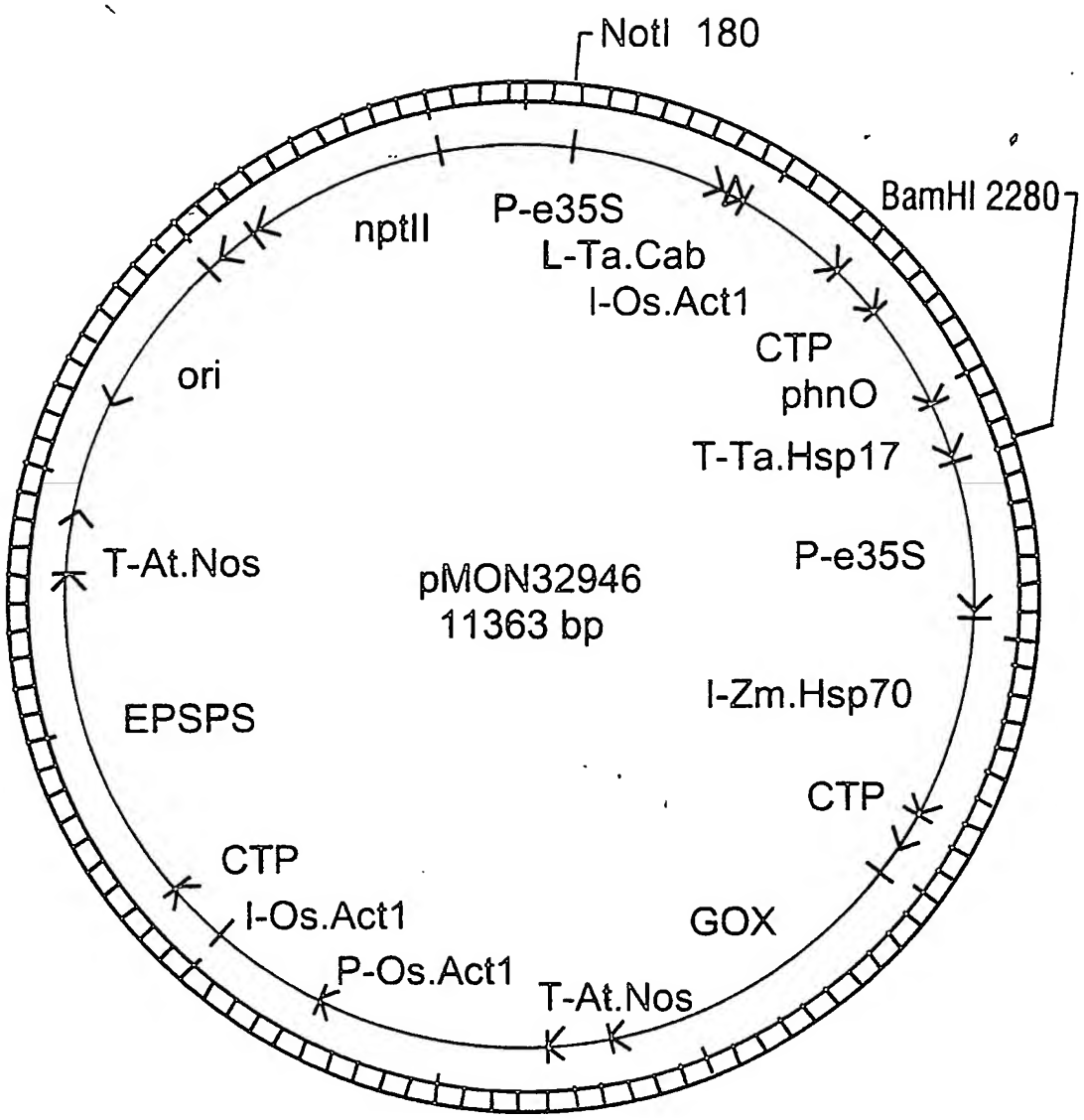
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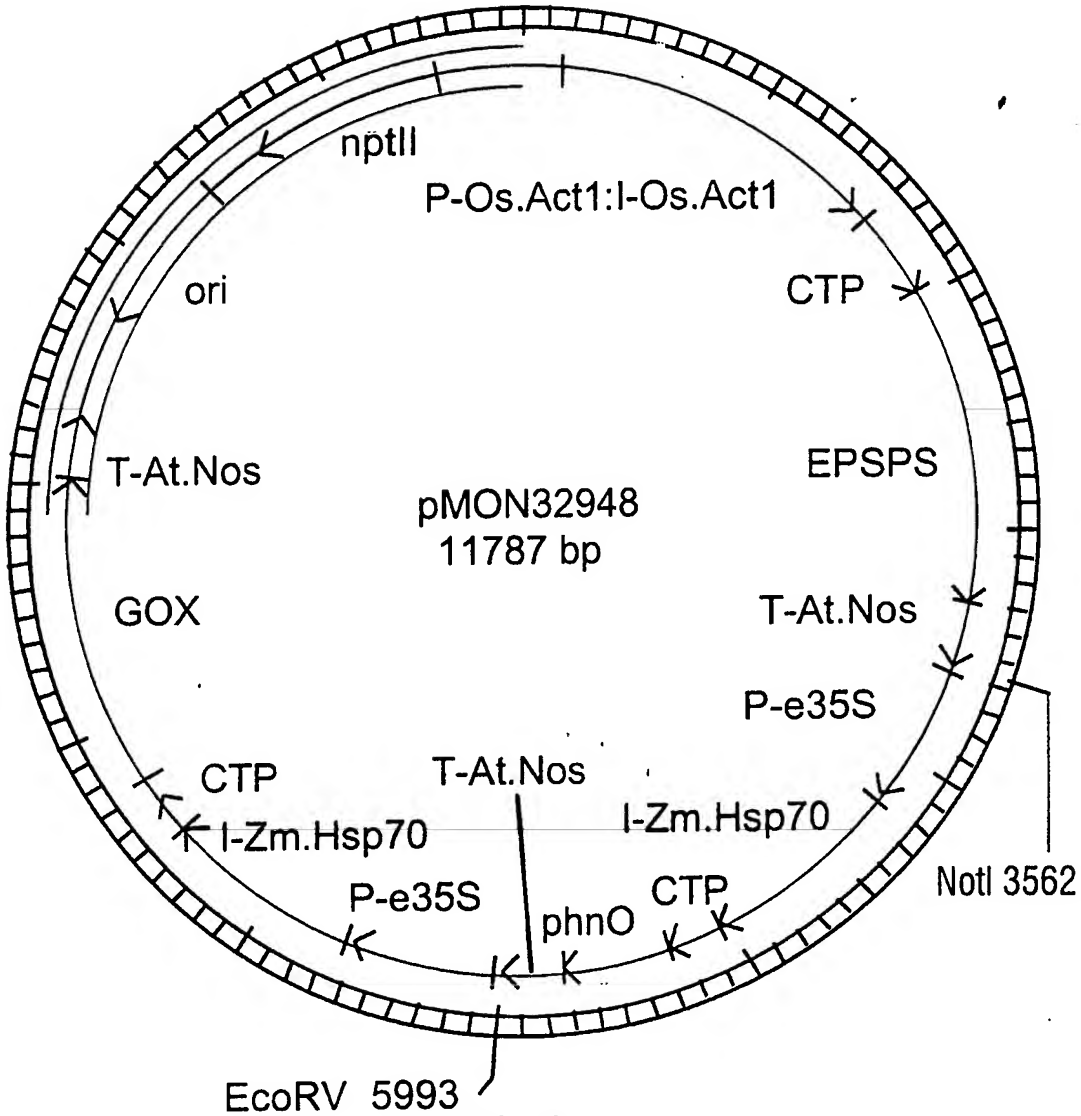
FIG. 7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NotI 3562
EcoRV 5993

FIG. 8

SEQUENCE LISTING

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<151> 1998-11-17

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tcatcctcca gttgccgacg ctcctgatca agctggcgct ggcgatcgtc cagctgcctg 11160

cggcgatctt caaactggcg gcggcggtcg tcatattgtc tgcgccgatc gtcgctcact 11220

tcacgctgcc agccgtcgtc gcgcgaatct tcatagtctc gcccacggtc agggttataa 11280

gcgtcattaa tcgcctgctg aatattgcca atggtgtcgt cgataatatc ggcctgggcc 11340

ggaacgtgga cagcgtgagc agggtgaata aaagaaatag cggaaagcgt ttcattagcc 11400

aacctcaaaa agaaactcta tccacattaa tcattactca tccatgcaag tagtggatga 11460

atctcaattt ctccgctgct ctattgccgt aatcgcctcc acgcgttgtt gatgacgacc 11520

gccttcgtac tgtgcgccca gccacgcatc cacaatcatt tttgccagtt cgaggccaac 11580

cactcgtgaa ccaaaagcca gcacgttggt gtcgttatgc tgccgcgaaa gttgcgcgga 11640

ataaggttcg ctacagacga ccgcgcgaat tc 11672

<211> 435
<212> DNA
<213> Escherichia coli

<400> 3
atgcctgctt gtgagcttcg cccggccacg cagtacgaca ccgacgcggt ttacgcgctg 60

atttgtgagc taaaacaggc ggagtttgac caccacgcgt ttcgcgtggg ttttaacgcc 120

aatctgcgcg acccaaacat gcgctaccat ctggcgctgc ttgatggcga agttgtcggc 180

atgatcggcc tgcatttgca gtttcatctg catcatgtca actggatcgg cgaaattcag 240

gagttggtgg taatgccgca ggcgcgcggt ctgaacgtcg gcagtaagtt actggcgtgg 300

gcagaagaag aagcccgcca ggccggggcc gaaatgaccg aactttcgac caacgtgaag 360

cgccacgacg cgcaccgttt ctatctgcgc gaaggctacg agcagagcca cttccgcttc 420

accaaggcgc tgtaa 435

<210> 4
<211> 144
<212> PRT
<213> Escherichia coli

<400> 4

Met Pro Ala Cys Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala
1 5 10 15

Val Tyr Ala Leu Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His
20 25 30

Ala Phe Arg Val Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg
35 40 45

Tyr His Leu Ala Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu
50 55 60

His Leu Gln Phe His Leu His His Val Asn Trp Ile Gly Glu Ile Gln
65 70 75 80

Glu Leu Val Val Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys
85 90 95

Leu Leu Ala Trp Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met
100 105 110

Thr Glu Leu Ser Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr
115 120 125

Leu Arg Glu Gly Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
130 135 140

<210> 5
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:synthetic oligonucleotide

<400> 5
aaacaccatg gctgcttgtg

<210> 6
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:synthetic oligonucleotide

<400> 6
gtgacgaatt cgagctcatt acagcgcctt ggtga 35

<210> 7
<211> 435
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:non-naturally occurring nucleotide sequence encoding modified PhnO protein P2A; g-c at nucleotide position 4

<220>
<221> CDS
<222> (1)..(432)

<400> 7

```
atg gct gct tgt gag ctt cgc ccg gcc acg cag tac gac acc gac gcg   48
Met Ala Ala Cys Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala
  1               5                  10                  15

gtt tac gcg ctg att tgt gag cta aaa cag gcg gag ttt gac cac cac   96
Val Tyr Ala Leu Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His
             20                  25                  30

gcg ttt cgc gtg ggt ttt aac gcc aat ctg cgc gac cca aac atg cgc   144
Ala Phe Arg Val Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg
         35                  40                  45

tac cat ctg gcg ctg ctt gat ggc gaa gtt gtc ggc atg atc ggc ctg   192
Tyr His Leu Ala Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu
     50                  55                  60

cat ttg cag ttt cat ctg cat cat gtc aac tgg atc ggc gaa att cag   240
His Leu Gln Phe His Leu His His Val Asn Trp Ile Gly Glu Ile Gln
 65                  70                  75                  80

gag ttg gtg gta atg ccg cag gcg cgc ggt ctg aac gtc ggc agt aag   288
Glu Leu Val Val Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys
                 85                  90                  95

tta ctg gcg tgg gca gaa gaa gaa gcc cgc cag gcc ggg gcc gaa atg   336
Leu Leu Ala Trp Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met
            100                 105                 110

acc gaa ctt tcg acc aac gtg aag cgc cac gac gcg cac cgt ttc tat   384
Thr Glu Leu Ser Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr
        115                 120                 125

ctg cgc gaa ggc tac gag cag agc cac ttc cgc ttc acc aag gcg ctg   432
Leu Arg Glu Gly Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
    130                 135                 140

taa                                                              435
```

<210> 8
<211> 144
<212> PRT

<213> Artificial Sequence

<400> 8

```
Met Ala Ala Cys Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala
 1               5                  10                  15

Val Tyr Ala Leu Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His
            20                  25                  30

Ala Phe Arg Val Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg
        35                  40                  45

Tyr His Leu Ala Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu
    50                  55                  60

His Leu Gln Phe His Leu His His Val Asn Trp Ile Gly Glu Ile
65                  70                  75                  80

Glu Leu Val Val Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys
                85                  90                  95

Leu Leu Ala Trp Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met
            100                 105                 110

Thr Glu Leu Ser Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr
        115                 120                 125

Leu Arg Glu Gly Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
    130                 135                 140
```

<210> 9
<211> 264
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:transit peptide coding sequence

<220>
<221> CDS
<222> (1)..(264)

<400> 9

```
atg gct tcc tct atg ctc tct tcc gct act atg gtt gcc tct ccg gct   48
Met Ala Ser Ser Met Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala
 1               5                  10                  15

cag gcc act atg gtc gct cct ttc aac gga ctt aag tcc tcc gct gcc   96
Gln Ala Thr Met Val Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala
            20                  25                  30

ttc cca gcc acc cgc aag gct aac aac gac att act tcc atc aca agc   144
Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser
        35                  40                  45
```

```
aac ggc gga aga gtt aac tgc atg cag gtg tgg cct ccg att gga aag   192
Asn Gly Gly Arg Val Asn Cys Met Gln Val Trp Pro Pro Ile Gly Lys
     50                  55                  60

aag aag ttt gag act ctc tct tac ctt cct gac ctt acc gat tcc ggt   240
Lys Lys Phe Glu Thr Leu Ser Tyr Leu Pro Asp Leu Thr Asp Ser Gly
 65                  70                  75                  80

ggt cgc gtc aac tgc atg cag gcc                                   264
Gly Arg Val Asn Cys Met Gln Ala
                 85
```

<210> 10
<211> 88
<212> PRT
<213> Artificial Sequence

<400> 10

```
Met Ala Ser Ser Met Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala
  1               5                  10                  15

Gln Ala Thr Met Val Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala
             20                  25                  30

Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser
         35                  40                  45

Asn Gly Gly Arg Val Asn Cys Met Gln Val Trp Pro Pro Ile Gly Lys
     50                  55                  60

Lys Lys Phe Glu Thr Leu Ser Tyr Leu Pro Asp Leu Thr Asp Ser Gly
 65                  70                  75                  80

Gly Arg Val Asn Cys Met Gln Ala
                 85
```

<210> 11
<211> 696
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: CTP-AMPA acetyltransferase coding sequence and amino acid sequence translation

<220>
<221> CDS
<222> (1)..(696)

<400> 11

```
atg gct tcc tct atg ctc tct tcc gct act atg gtt gcc tct ccg gct   48
Met Ala Ser Ser Met Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala
  1               5                  10                  15
```

```
cag gcc act atg gtc gct cct ttc aac gga ctt aag tcc tcc gct gcc   96
Gln Ala Thr Met Val Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala
             20                  25                  30

ttc cca gcc acc cgc aag gct aac aac gac att act tcc atc aca agc  144
Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser
         35                  40                  45

aac ggc gga aga gtt aac tgc atg cag gtg tgg cct ccg att gga aag  192
Asn Gly Gly Arg Val Asn Cys Met Gln Val Trp Pro Pro Ile Gly Lys
     50                  55                  60

aag aag ttt gag act ctc tct tac ctt cct gac ctt acc gat tcc ggt  240
Lys Lys Phe Glu Thr Leu Ser Tyr Leu Pro Asp Leu Thr Asp Ser Gly
 65                  70                  75                  80

ggt cgc gtc aac tgc atg cag gcc atg gct gct tgt gag ctt cgc ccg  288
Gly Arg Val Asn Cys Met Gln Ala Met Ala Ala Cys Glu Leu Arg Pro
                 85                  90                  95

gcc acg cag tac gac acc gac gcg gtt tac gcg ctg att tgt gag cta  336
Ala Thr Gln Tyr Asp Thr Asp Ala Val Tyr Ala Leu Ile Cys Glu Leu
            100                 105                 110

aaa cag gcg gag ttt gac cac cac gcg ttt cgc gtg ggt ttt aac gcc  384
Lys Gln Ala Glu Phe Asp His His Ala Phe Arg Val Gly Phe Asn Ala
        115                 120                 125

aat ctg cgc gac cca aac atg cgc tac cat ctg gcg ctg ctt gat ggc  432
Asn Leu Arg Asp Pro Asn Met Arg Tyr His Leu Ala Leu Leu Asp Gly
    130                 135                 140

gaa gtt gtc ggc atg atc ggc ctg cat ttg cag ttt cat ctg cat cat  480
Glu Val Val Gly Met Ile Gly Leu His Leu Gln Phe His Leu His His
145                 150                 155                 160

gtc aac tgg atc ggc gaa att cag gag ttg gtg gta atg ccg cag gcg  528
Val Asn Trp Ile Gly Glu Ile Gln Glu Leu Val Val Met Pro Gln Ala
                165                 170                 175

cgc ggt ctg aac gtc ggc agt aag tta ctg gcg tgg gca gaa gaa gaa  576
Arg Gly Leu Asn Val Gly Ser Lys Leu Leu Ala Trp Ala Glu Glu Glu
            180                 185                 190

gcc cgc cag gcc ggg gcc gaa atg acc gaa ctt tcg acc aac gtg aag  624
Ala Arg Gln Ala Gly Ala Glu Met Thr Glu Leu Ser Thr Asn Val Lys
        195                 200                 205

cgc cac gac gcg cac cgt ttc tat ctg cgc gaa ggc tac gag cag agc  672
Arg His Asp Ala His Arg Phe Tyr Leu Arg Glu Gly Tyr Glu Gln Ser
    210                 215                 220

cac ttc cgc ttc acc aag gcg ctg                                 696
His Phe Arg Phe Thr Lys Ala Leu
225                 230
```

<210> 12
<211> 232
<212> PRT
<213> Artificial Sequence

<400> 12

```
Met Ala Ser Ser Met Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala
 1               5                  10                  15

Gln Ala Thr Met Val Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala
            20                  25                  30

Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser
        35                  40                  45

Asn Gly Gly Arg Val Asn Cys Met Gln Val Trp Pro Pro Ile Gly Lys
    50                  55                  60

Lys Lys Phe Glu Thr Leu Ser Tyr Leu Pro Asp Leu Thr Asp Ser Gly
65                  70                  75                  80

Gly Arg Val Asn Cys Met Gln Ala Met Ala Ala Cys Glu Leu Arg Pro
                85                  90                  95

Ala Thr Gln Tyr Asp Thr Asp Ala Val Tyr Ala Leu Ile Cys Glu Leu
            100                 105                 110

Lys Gln Ala Glu Phe Asp His His Ala Phe Arg Val Gly Phe Asn Ala
        115                 120                 125

Asn Leu Arg Asp Pro Asn Met Arg Tyr His Leu Ala Leu Leu Asp Gly
    130                 135                 140

Glu Val Val Gly Met Ile Gly Leu His Leu Gln Phe His Leu His His
145                 150                 155                 160

Val Asn Trp Ile Gly Glu Ile Gln Glu Leu Val Val Met Pro Gln Ala
                165                 170                 175

Arg Gly Leu Asn Val Gly Ser Lys Leu Leu Ala Trp Ala Glu Glu Glu
            180                 185                 190

Ala Arg Gln Ala Gly Ala Glu Met Thr Glu Leu Ser Thr Asn Val Lys
        195                 200                 205

Arg His Asp Ala His Arg Phe Tyr Leu Arg Glu Gly Tyr Glu Gln Ser
    210                 215                 220

His Phe Arg Phe Thr Lys Ala Leu
225                 230
```

<210> 13
<211> 415
<212> DNA
<213> Zea mays

<220>
<221> N_region
<222> (15)..(163)

<220>
<221> intron
<222> (164)..(322)

<220>
<221> C_region
<222> (323)..(411)

<400> 13
tctagaggat cagcatggcg cccaccgtga tgatggcctc gtcggccacc gccgtcgctc 60

cgttcctggg gctcaagtcc accgccagcc tccccgtcgc ccgccgctcc tccagaagcc 120

tcggcaacgt cagcaacggc ggaaggatcc ggtgcatgca ggtaacaaat gcatcctagc 180

tagtagttct ttgcattgca gcagctgcag ctagcgagtt agtaatagga agggaactga 240

tgatccatgc atggactgat gtgtgttgcc catcccatcc catcccattt cccaaacgaa 300

ccgaaaacac cgtactacgt gcaggtgtgg ccctacggca acaagaagtt cgagacgctg 360

tcgtacctgc cgccgctgtc gaccggcggg cgcatccgct gcatgcaggc catgg 415

<210> 14
<211> 174
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: chloroplast or plastid transit peptide coding sequence and amino acid sequence translation

<220>
<221> CDS
<222> (1)..(174)

<400> 14
atg gct tcc tct atg ctc tct tcc gct act atg gtt gcc tct ccg gct 48
Met Ala Ser Ser Met Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala
1 5 10 15

cag gcc act atg gtc gct cct ttc aac gga ctt aag tcc tcc gct gcc 96
Gln Ala Thr Met Val Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala
20 25 30

ttc cca gcc acc cgc aag gct aac aac gac att act tcc atc aca agc 144
Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser
35 40 45

aac ggc gga aga gtt aac tgc atg cag gcc 174
Asn Gly Gly Arg Val Asn Cys Met Gln Ala

50 55

<210> 15
<211> 58
<212> PRT
<213> Artificial Sequence

<400> 15
Met Ala Ser Ser Met Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala
1 5 10 15

Gln Ala Thr Met Val Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala
20 25 30

Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser
35 40 45

Asn Gly Gly Arg Val Asn Cys Met Gln Ala
50 55

<210> 16
<211> 157
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide representing base pairs 1 through 157 of a 432 base pair AMPA acyltransferase gene

<400> 16
atggccgctt gcgagcttcg cccagccacg cagtacgaca ccgacgccgt gtacgcgctg 60

atctgcgagc tcaagcaggc ggagttcgac caccacgcct tccgcgtggg cttcaacgcc 120

aacctgcgcg accccaacat gcgctaccat ctggcgc 157

<210> 17
<211> 187
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide sequence representing base pairs 158 through 344 of a 432 base pair AMPA acyltransferase gene

<400> 17
tgcttgatgg cgaagtggtc ggcatgatcg gcctgcacct ccagttccac ctgcatcatg 60

tcaactggat cggcgagatc caggagctgg tcgtgatgcc acaggcgagg ggtctgaacg 120

tcggcagcaa gctcctggcg tgggccgagg aggaagccag gcaggccgga gccgagatga 180

ccgagct 187

<210> 18
<211> 88
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide sequence representing base pairs 345 through 432 of a 432 base pair AMPA acyltransferase gene

<400> 18
cagcaccaac gtgaagcgcc acgacgcgca ccgcttctac ctgcgcgaag gctacgagca 60

gagccacttc cgcttcacca aggcgctg 88

<210> 19
<211> 432
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide providing monocot optimized coding sequence for an AMPA acetyltransferase

<220>
<221> CDS
<222> (1)..(432)

<400> 19
atg gcc gct tgc gag ctt cgc cca gcc acg cag tac gac acc gac gcc 48
Met Ala Ala Cys Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala
1 5 10 15

gtg tac gcg ctg atc tgc gag ctc aag cag gcg gag ttc gac cac cac 96
Val Tyr Ala Leu Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His
20 25 30

gcc ttc cgc gtg ggc ttc aac gcc aac ctg cgc gac ccc aac atg cgc 144
Ala Phe Arg Val Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg
35 40 45

tac cat ctg gcg ctg ctt gat ggc gaa gtg gtc ggc atg atc ggc ctg 192
Tyr His Leu Ala Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu
50 55 60

cac ctc cag ttc cac ctg cat cat gtc aac tgg atc ggc gag atc cag 240
His Leu Gln Phe His Leu His His Val Asn Trp Ile Gly Glu Ile Gln
65 70 75 80

gag ctg gtc gtg atg cca cag gcg agg ggt ctg aac gtc ggc agc aag 288

```
Glu Leu Val Val Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys
                 85                  90                  95

ctc ctg gcg tgg gcc gag gag gaa gcc agg cag gcc gga gcc gag atg   336
Leu Leu Ala Trp Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met
            100                 105                 110

acc gag ctc agc acc aac gtg aag cgc cac gac gcg cac cgc ttc tac   384
Thr Glu Leu Ser Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr
        115                 120                 125

ctg cgc gaa ggc tac gag cag agc cac ttc cgc ttc acc aag gcg ctg   432
Leu Arg Glu Gly Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
    130                 135                 140
```

<210> 20
<211> 144
<212> PRT
<213> Artificial Sequence

<400> 20

```
Met Ala Ala Cys Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala
  1               5                  10                  15

Val Tyr Ala Leu Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His
             20                  25                  30

Ala Phe Arg Val Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg
         35                  40                  45

Tyr His Leu Ala Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu
     50                  55                  60

His Leu Gln Phe His Leu His His Val Asn Trp Ile Gly Glu Ile Gln
 65                  70                  75                  80

Glu Leu Val Val Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys
                 85                  90                  95

Leu Leu Ala Trp Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met
            100                 105                 110

Thr Glu Leu Ser Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr
        115                 120                 125

Leu Arg Glu Gly Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
    130                 135                 140
```

<210> 21
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic

oligonucleotide PHN1 for use as an amplification primer

<400> 21
atggctgctt gtgagcttcg

<210> 22
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide PHN2 for use as an amplification primer

<400> 22
cagcgccttg gtgaagcgga

<210> 23
<211> 1630
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: expression cassette comprising plant operable promoter linked to a coding sequence encoding an AMPA acetyltransferase linked to a transcription termination sequence

<220>
<221> promoter
<222> (33)..(605)

<220>
<221> transit_peptide
<222> (627)..(892)

<220>
<221> CDS
<222> (893)..(1324)

<220>
<221> terminator
<222> (1350)..(1605)

<400> 23
gcggccgcgt tcaagcttga gctcaggatt tagcagcatt ccagattggg ttcaatcaac 60

aaggtacgag ccatatcact ttattcaaat tggtatcgcc aaaaccaaga aggaactccc 120

atcctcaaag gtttgtaagg aagaattctc agtccaaagc ctcaacaagg tcagggtaca 180

gagtctccaa accattagcc aaaagctaca ggagatcaat gaagaatctt caatcaaagt 240

aaactactgt tccagcacat gcatcatggt cagtaagttt cagaaaaaga catccaccga 300

agacttaaag ttagtgggca tctttgaaag taatcttgtc aacatcgagc agctggcttg 360

tgcgcaccag acaaaaaagg aatggtgcag aattgttagg cgcacctacc aaaagcatct 420

ttgcctttat tgcaaagata aagcagattc ctctagtaca agtggggaac aaaataacgt 480

ggaaaagagc tgtcctgaca gcccactcac taatgcgtat gacgaacgca gtgacgacca 540

caaaagaatt ccctctatat aagaaggcat tcattcccat ttgaaggatc atcagatact 600

gaaccaatcc ttctagaaga tctccacaat ggcttcctct atgctctctt ccgctactat 660

ggttgcctct ccggctcagg ccactatggt cgctcctttc aacggactta agtcctccgc 720

tgccttccca gccacccgca aggctaacaa cgacattact tccatcacaa gcaacggcgg 780

aagagttaac tgcatgcagg tgtggcctcc gattggaaag aagaagtttg agactctctc 840

ttaccttcct gaccttaccg attccggtgg tcgcgtcaac tgcatgcagg cc atg gct 898
Met Ala

gct tgt gag ctt cgc ccg gcc acg cag tac gac acc gac gcg gtt tac 946
Ala Cys Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala Val Tyr
5 10 15

gcg ctg att tgt gag cta aaa cag gcg gag ttt gac cac cac gcg ttt 994
Ala Leu Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His Ala Phe
20 25 30

cgc gtg ggt ttt aac gcc aat ctg cgc gac cca aac atg cgc tac cat 1042
Arg Val Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg Tyr His
35 40 45 50

ctg gcg ctg ctt gat ggc gaa gtt gtc ggc atg atc ggc ctg cat ttg 1090
Leu Ala Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu His Leu
55 60 65

cag ttt cat ctg cat cat gtc aac tgg atc ggc gaa att cag gag ttg 1138
Gln Phe His Leu His His Val Asn Trp Ile Gly Glu Ile Gln Glu Leu
70 75 80

gtg gta atg ccg cag gcg cgc ggt ctg aac gtc ggc agt aag tta ctg 1186
Val Val Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys Leu Leu
85 90 95

gcg tgg gca gaa gaa gaa gcc cgc cag gcc ggg gcc gaa atg acc gaa 1234
Ala Trp Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met Thr Glu
100 105 110

ctt tcg acc aac gtg aag cgc cac gac gcg cac cgt ttc tat ctg cgc 1282
Leu Ser Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr Leu Arg
115 120 125 130

gaa ggc tac gag cag agc cac ttc cgc ttc acc aag gcg ctg 1324
Glu Gly Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
135 140

taatgagctc ggtaccggat ccaattcccg atcgttcaaa catttggcaa taaagtttct 1384

taagattgaa tcctgttgcc ggtcttgcga tgattatcat ataatttctg ttgaattacg 1444

ttaagcatgt aataattaac atgtaatgca tgacgttatt tatgagatgg gtttttatga 1504

ttagagtccc gcaattatac atttaatacg cgatagaaaa caaaatatag cgcgcaaact 1564

aggataaatt atcgcgcgcg gtgtcatcta tgttactaga tcggggatcg atccccgggc 1624

ggccgc 1630

<210> 24
<211> 144
<212> PRT
<213> Artificial Sequence

<400> 24
Met Ala Ala Cys Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala
1 5 10 15

Val Tyr Ala Leu Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His
20 25 30

Ala Phe Arg Val Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg
35 40 45

Tyr His Leu Ala Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu
50 55 60

His Leu Gln Phe His Leu His His Val Asn Trp Ile Gly Glu Ile Gln
65 70 75 80

Glu Leu Val Val Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys
85 90 95

Leu Leu Ala Trp Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met
100 105 110

Thr Glu Leu Ser Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr
115 120 125

Leu Arg Glu Gly Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
130 135 140

<210> 25
<211> 2122
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:expression cassette comprising plant promoter linked to sequence encoding AMPA acetyl transferase linked to termination sequence

<220>
<221> promoter
<222> (6)..(620)

<220>
<221> 5'UTR
<222> (645)..(715)

<220>
<221> intron
<222> (729)..(1178)

<220>
<221> transit_peptide
<222> (1179)..(1406)

<220>
<221> CDS
<222> (1407)..(1838)

<220>
<221> terminator
<222> (1849)..(2082)

<400> 25
ctgcaggtcc gatgtgagac ttttcaacaa agggtaatat ccggaaacct cctcggattc 60

cattgcccag ctatctgtca ctttattgtg aagatagtgg aaaaggaagg tggctcctac 120

aaatgccatc attgcgataa aggaaaggcc atcgttgaag atgcctctgc cgacagtggt 180

cccaaagatg gacccccacc cacgaggagc atcgtggaaa aagaagacgt tccaaccacg 240

tcttcaaagc aagtggattg atgtgatggt ccgatgtgag acttttcaac aaagggtaat 300

atccggaaac ctcctcggat tccattgccc agctatctgt cactttattg tgaagatagt 360

ggaaaaggaa ggtggctcct acaaatgcca tcattgcgat aaaggaaagg ccatcgttga 420

agatgcctct gccgacagtg gtcccaaaga tggaccccca cccacgagga gcatcgtgga 480

aaaagaagac gttccaacca cgtcttcaaa gcaagtggat tgatgtgata tctccactga 540

cgtaagggat gacgcacaat cccactatcc ttcgcaagac ccttcctcta tataaggaag 600

ttcatttcat ttggagagga cacgctgaca agctgactct agcagatcct ctagaaccat 660

cttccacaca ctcaagccac actattggag aacacacagg gacaacacac cataagatcc 720

aagggaggcc tccgccgccg ccggtaacca ccccgccctc ctcctctttc tttctccgtt 780

ttttttccg tctcggtctc gatctttggc cttggtagtt tgggtgggcg agaggcggct 840

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tcgtgcgcgc ccaga.cggt gcgcgggagg ggcgggatct cgcggggaat ggggctctcg 900

gatgtagatc tgcgatccgc cgttgttggg ggagatgatg gggcgtttaa aatttcgccg 960

tgctaaacaa gatcaggaag aggggaaaag ggcactatgg tttatatttt tatatatttc 1020

tgctgcttcg tcaggcttag atgtgctaga tctttctttc ttctttttgt gggtagaatt 1080

taatccctca gcattgttca tcggtagttt ttcttttcat gatttcgtga caaatgcagc 1140

ctcgtgcgga gctttttttgt aggtagaagt gatcaaccat ggcgcaagtt agcagaatct 1200

gcaatggtgt gcagaaccca tctcttatct ccaatctctc gaaatccagt caacgcaaat 1260

ctcccttatc ggtttctctg aagacgcagc agcatccacg agcttatccg atttcgtcgt 1320

cgtggggatt gaagaagagt gggatgacgt taattggctc tgagcttcgt cctcttaagg 1380

tcatgtcttc tgtttccacg gcgtgc atg gcc gct tgc gag ctt cgc cca gcc  1433
                            Met Ala Ala Cys Glu Leu Arg Pro Ala

acg cag tac gac acc gac gcc gtg tac gcg ctg atc tgc gag ctc aag   1481
Thr Gln Tyr Asp Thr Asp Ala Val Tyr Ala Leu Ile Cys Glu Leu Lys
 10                  15                  20                  25

cag gcg gag ttc gac cac cac gcc ttc cgc gtg ggc ttc aac gcc aac   1529
Gln Ala Glu Phe Asp His His Ala Phe Arg Val Gly Phe Asn Ala Asn
                 30                  35                  40

ctg cgc gac ccc aac atg cgc tac cat ctg gcg ctg ctt gat ggc gaa   1577
Leu Arg Asp Pro Asn Met Arg Tyr His Leu Ala Leu Leu Asp Gly Glu
             45                  50                  55

gtg gtc ggc atg atc ggc ctg cac ctc cag ttc cac ctg cat cat gtc   1625
Val Val Gly Met Ile Gly Leu His Leu Gln Phe His Leu His His Val
         60                  65                  70

aac tgg atc ggc gag atc cag gag ctg gtc gtg atg cca cag gcg agg   1673
Asn Trp Ile Gly Glu Ile Gln Glu Leu Val Val Met Pro Gln Ala Arg
     75                  80                  85

ggt ctg aac gtc ggc agc aag ctc ctg gcg tgg gcc gag gag gaa gcc   1721
Gly Leu Asn Val Gly Ser Lys Leu Leu Ala Trp Ala Glu Glu Glu Ala
 90                  95                  100                 105

agg cag gcc gga gcc gag atg acc gag ctc agc acc aac gtg aag cgc   1769
Arg Gln Ala Gly Ala Glu Met Thr Glu Leu Ser Thr Asn Val Lys Arg
                110                 115                 120

cac gac gcg cac cgc ttc tac ctg cgc gaa ggc tac gag cag agc cac   1817
His Asp Ala His Arg Phe Tyr Leu Arg Glu Gly Tyr Glu Gln Ser His
            125                 130                 135

ttc cgc ttc acc aag gcg ctg taaagatctg aattctgcat gcgtttggac      1868
Phe Arg Phe Thr Lys Ala Leu
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140

gtatgctcat tcaggttgga gccaatttgg ttgatgtgtg tgcgagttct tgcgagtctg 1928

atgagacatc tctgtattgt gtttctttcc ccagtgtttt ctgtacttgt gtaatcggct 1988

aatcgccaac agattcggcg atgaataaat gagaaataaa ttgttctgat tttgagtgca 2048

aaaaaaaagg aattagatct gtgtgtgttt tttggatccc cggggcggcc gccccgggtg 2108

gtgagcttct gcag

<210> 26
<211> 144
<212> PRT
<213> Artificial Sequence

<400> 26

Met Ala Ala Cys Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala
1 5 10 15

Val Tyr Ala Leu Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His
20 25 30

Ala Phe Arg Val Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg
35 40 45

Tyr His Leu Ala Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu
50 55 60

His Leu Gln Phe His Leu His His Val Asn Trp Ile Gly Glu Ile Gln
65 70 75 80

Glu Leu Val Val Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys
85 90 95

Leu Leu Ala Trp Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met
100 105 110

Thr Glu Leu Ser Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr
115 120 125

Leu Arg Glu Gly Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
130 135 140

<210> 27
<211> 2378
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:expression cassette comprising a plant promoter linked to an intron, a sequence encoding an AMPA acetyl transferase, and a termination sequence

<220>
<221> promoter
<222> (28)..(965)

<220>
<221> intron
<222> (966)..(1423)

<220>
<221> transit_peptide
<222> (1440)..(1667)

<220>
<221> CDS
<222> (1668)..(2099)

<220>
<221> terminator
<222> (2114)..(2369)

<400> 27
gatatcccta gggcggccgc gttaacaagc ttactcgagg tcattcatat gcttgagaag 60

agagtcggga tagtccaaaa taaaacaaag gtaagattac ctggtcaaaa gtgaaaacat 120

cagttaaaag gtggtataaa gtaaaatatc ggtaataaaa ggtggcccaa agtgaaattt 180

actcttttct actattataa aaattgagga tgtttttgtc ggtactttga tacgtcattt 240

ttgtatgaat tggtttttaa gtttattcgc ttttggaaat gcatatctgt atttgagtcg 300

ggttttaagt tcgtttgctt ttgtaaatac agagggattt gtataagaaa tatctttaga 360

aaaacccata tgctaatttg acataatttt tgagaaaaat atatattcag gcgaattctc 420

acaatgaaca ataataagat taaaatagct ttcccccgtt gcagcgcatg ggtatttttt 480

ctagtaaaaa taaaagataa acttagactc aaaacattta caaaaacaac ccctaaagtt 540

cctaaagccc aaagtgctat ccacgatcca tagcaagccc agcccaaccc aacccaaccc 600

agcccacccc agtccagcca actggacaat agtctccaca ccccccccact atcaccgtga 660

gttgtccgca cgcaccgcac gtctcgcagc caaaaaaaaa aagaaagaaa aaaaagaaaa 720

agaaaaaaca gcaggtgggt ccgggtcgtg ggggccggaa acgcgaggag gatcgcgagc 780

cagcgacgag gccggccctc cctccgcttc caaagaaacg ccccccatcg ccactatata 840

catacccccc cctctcctcc catccccccca accctaccac caccaccacc accacctcca 900

cctcctcccc cctcgctgcc ggacgacgag ctcctccccc ctccccctcc gccgccgccg 960

cgccggtaac caccccgccc ctctcctctt tctttctccg ttttttttttc cgtctcggtc 1020

tcgatctttg gccttggtag tttgggtggg cgagaggcgg cttcgtgccg cccagatcgg 1080

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tgcgcgggag gggcgggatc tcgcggctgg ctctcgcccc cgtggatccg gcccggatct 1140

cgcggggaat ggggctctcg gatgtagatc tgcgatccgc cgttgttggg gccgatgatg 1200

gggcccttaa aatttccgcc gtgctaaaca agatcaggaa gaggggaaaa gggcactatg 1260

gtttatattt ttatatattt ctgctgcttc gtcaggctta gatgtgctag atctttcttt 1320

cttctttttg tgggtagaat ttaatccctc agcattgttc atcggtagtt tttcttttca 1380

tgattcgtga caaatgcagc ctcgtgcgga cgttttttg taggtagaag tgatcaacca 1440

tggcgcaagt tagcagaatc tgcaatggtg tgcagaaccc atctcttatc tccaatctct 1500

cgaaatccag tcaacgcaaa tctcccttat cggtttctct gaagacgcag cagcatccac 1560

gagcttatcc gatttcgtcg tcgtggggat tgaagaagag tgggatgacg ttaattggct 1620

ctgagcttcg tcctcttaag gtcatgtctt ctgtttccac ggcgtgc atg gcc gct   1676
                                                Met Ala Ala

tgc gag ctt cgc cca gcc acg cag tac gac acc gac gcc gtg tac gcg   1724
Cys Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala Val Tyr Ala
      5                  10                  15

ctg atc tgc gag ctc aag cag gcg gag ttc gac cac cac gcc ttc cgc   1772
Leu Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His Ala Phe Arg
 20                  25                  30                  35

gtg ggc ttc aac gcc aac ctg cgc gac ccc aac atg cgc tac cat ctg   1820
Val Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg Tyr His Leu
                 40                  45                  50

gcg ctg ctt gat ggc gaa gtg gtc ggc atg atc ggc ctg cac ctc cag   1868
Ala Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu His Leu Gln
            55                  60                  65

ttc cac ctg cat cat gtc aac tgg atc ggc gag atc cag gag ctg gtc   1916
Phe His Leu His His Val Asn Trp Ile Gly Glu Ile Gln Glu Leu Val
        70                  75                  80

gtg atg cca cag gcg agg ggt ctg aac gtc ggc agc aag ctc ctg gcg   1964
Val Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys Leu Leu Ala
     85                  90                  95

tgg gcc gag gag gaa gcc agg cag gcc gga gcc gag atg acc gag ctc   2012
Trp Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met Thr Glu Leu
100                 105                 110                 115

agc acc aac gtg aag cgc cac gac gcg cac cgc ttc tac ctg cgc gaa   2060
Ser Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr Leu Arg Glu
                120                 125                 130

ggc tac gag cag agc cac ttc cgc ttc acc aag gcg ctg taaagatctg    2109
Gly Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
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135 140

aattcccgat cgttcaaaca tttggcaata aagtttctta agattgaatc ctgttgccgg 2169

tcttgcgatg attatcatat aatttctgtt gaattacgtt aagcatgtaa taattaacat 2229

gtaatgcatg acgttattta tgagatgggt ttttatgatt agagtcccgc aattatacat 2289

ttaatacgcg atagaaaaca aaatatagcg cgcaaactag gataaattat cgcgcgcggt 2349

gtcatctatg ttactagatc ggggatatc 2378

<210> 28
<211> 144
<212> PRT
<213> Artificial Sequence

<400> 28

Met Ala Ala Cys Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala
1 5 10 15

Val Tyr Ala Leu Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His
20 25 30

Ala Phe Arg Val Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg
35 40 45

Tyr His Leu Ala Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu
50 55 60

His Leu Gln Phe His Leu His His Val Asn Trp Ile Gly Glu Ile Gln
65 70 75 80

Glu Leu Val Val Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys
85 90 95

Leu Leu Ala Trp Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met
100 105 110

Thr Glu Leu Ser Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr
115 120 125

Leu Arg Glu Gly Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
130 135 140

<210> 29
<211> 2107
<212> DNA
<213> Artificial

<220>
<223> Description of Artificial Sequence:expression cassette comprising plant operable promoter linked to a leader, intron, a sequence encoding an AMPA acetyltransferase, and termination sequence

<220>
<221> promoter
<222> (26)..(590)

<220>
<221> 5'UTR
<222> (615)..(685)

<220>
<221> intron
<222> (699)..(1148)

<220>
<221> transit_peptide
<222> (1149)..(1426)

<220>
<221> CDS
<222> (1427)..(1858)

<220>
<221> terminator
<222> (1869)..(2102)

<400> 29
gcggccgcgt taacaagctt ctgcaggtcc gatgtgagac ttttcaacaa agggtaatat 60

ccggaaacct cctcggattc cattgcccag ctatctgtca ctttattgtg aagatagtgg 120

aaaaggaagg tggctcttac aaatgccatc attgcgataa aggaaaggcc atcgttgaag 180

atgcctctgc cgacagtggt cccaaagatg gacccccacc cacgaggagc atcgtggaaa 240

aagaagacgt tccaaccacg tcttcaaagc aagtggattg atgtgatggt ccgatgtgag 300

acttttcaac aaagggtaat atccggaaac ctcctcggat tccattgccc agctatctgt 360

cactttattg tgaagatagt ggaaaaggaa ggtggctcct acaaatgcca tcattgcgat 420

aaaggaaagg ccatcgttga agatgcctct gccgacagtg gtcccaaaga tggaccccca 480

cccacgagga gcatcgtgga aaaagaagac gttccaacca cgtcttcaaa gcaagtggat 540

tgatgtgata tctccactga cgtaagggat gacgcacaat cccactatcc ttcgcaagac 600

ccttcctcta tataaggaag ttcatttcat ttggagagga cacgctgaca agctgactct 660

agcagatcct ctagaaccat cttccacaca ctcaagccac actattggag aacacacagg 720

gacaacacac cataagatcc aagggaggcc tccgccgccg ccggtaacca ccccgccctt 780

ctcctctttc tttctccgtt ttttttccg tctcggtctc gatctttggc cttggtagtt 840

tgggtgggcg agaggcggct tcgtgcgcgc ccagatcggt gcgcgggagg ggcggatct 900

cgcggggaat ggggctctcg gatgtagatc tgcgatccgc cgttgttggg ggagatgatg 960

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gggcgtttaa aatttcgccg tgctaaacaa gatcaggaag aggggaaaag ggcactatgg 1020

tttatatttt tatatatttc tgctgcttcg tcaggcttag atgtgctaga tctttctttc 1080

ttctttttgt gggtagaatt taatccctca gcattgttca tcggtagttt ttcttttcat 1140

gatttcgtga caaatgcagc ctcgtgcgga gctttttgt aggtagaagt gatcaaccat 1200

ggcgcaagtt agcagaatct gcaatggtgt gcagaaccca tctcttatct ccaatctctc 1260

gaaatccagt caacgcaaat ctcccttatc ggtttctctg aagacgcagc agcatccacg 1320

agcttatccg atttcgtcgt cgtggggatt gaagaagagt gggatgacgt taattggctc 1380

tgagcttcgt cctcttaagg tcatgtcttc tgtttccacg gcgtgc atg gcc gct    1435
                                                Met Ala Ala

tgc gag ctt cgc cca gcc acg cag tac gac acc gac gcc gtg tac gcg    1483
Cys Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala Val Tyr Ala
      5                  10                  15

ctg atc tgc gag ctc aag cag gcg gag ttc gac cac cac gcc ttc cgc    1531
Leu Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His Ala Phe Arg
 20                  25                  30                  35

gtg ggc ttc aac gcc aac ctg cgc gac ccc aac atg cgc tac cat ctg    1579
Val Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg Tyr His Leu
                 40                  45                  50

gcg ctg ctt gat ggc gaa gtg gtc ggc atg atc ggc ctg cac ctc cag    1627
Ala Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu His Leu Gln
             55                  60                  65

ttc cac ctg cat cat gtc aac tgg atc ggc gag atc cag gag ctg gtc    1675
Phe His Leu His His Val Asn Trp Ile Gly Glu Ile Gln Glu Leu Val
         70                  75                  80

gtg atg cca cag gcg agg ggt ctg aac gtc ggc agc aag ctc ctg gcg    1723
Val Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys Leu Leu Ala
     85                  90                  95

tgg gcc gag gag gaa gcc agg cag gcc gga gcc gag atg acc gag ctc    1771
Trp Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met Thr Glu Leu
100                 105                 110                 115

agc acc aac gtg aag cgc cac gac gcg cac cgc ttc tac ctg cgc gaa    1819
Ser Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr Leu Arg Glu
                120                 125                 130

ggc tac gag cag agc cac ttc cgc ttc acc aag gcg ctg taaagatctg    1868
Gly Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
            135                 140

aattctgcat gcgtttggac gtatgctcat tcaggttgga gccaatttgg ttgatgtgtg 1928
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tgcgagttct tgcgagtctg atgagacatc tctgtattgt gtttctttcc ccagtgtttt 1988

ctgtacttgt gtaatcggct aatcgccaac agattcggcg atgaataaat gagaaataaa 2048

ttgttctgat tttgagtgca aaaaaaaagg aattagatct gtgtgtgttt tttggatcc 2107

<210> 30
<211> 144
<212> PRT
<213> Artificial Sequence

<400> 30
Met Ala Ala Cys Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala
1 5 10 15

Val Tyr Ala Leu Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His
20 25 30

Ala Phe Arg Val Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg
35 40 45

Tyr His Leu Ala Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu
50 55 60

His Leu Gln Phe His Leu His His Val Asn Trp Ile Gly Glu Ile Gln
65 70 75 80

Glu Leu Val Val Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys
85 90 95

Leu Leu Ala Trp Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met
100 105 110

Thr Glu Leu Ser Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr
115 120 125

Leu Arg Glu Gly Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
130 135 140

<210> 31
<211> 2436
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:monocot expression cassette comprising plant operable promoter linked to an intron, a sequence coding for an AMPA acetyltransferase, and a termination sequence

<220>
<221> promoter
<222> (26)..(640)

<220>
<221> intron
<222> (670)..(1473)

<220>
<221> transit_peptide
<222> (1498)..(1725)

<220>
<221> CDS
<222> (1726)..(2157)

<220>
<221> terminator
<222> (2172)..(2427)

<400> 31
gcggccgcgt taacaagctt ctgcaggtcc gatgtgagac ttttcaacaa agggtaatat 60

ccggaaacct cctcggattc cattgcccag ctatctgtca ctttattgtg aagatagtgg 120

aaaaggaagg tggctcctac aaatgccatc attgcgataa aggaaaggcc atcgttgaag 180

atgcctctgc cgacagtggt cccaaagatg gacccccacc cacgaggagc atcgtggaaa 240

aagaagacgt tccaaccacg tcttcaaagc aagtggattg atgtgatggt ccgatgtgag 300

acttttcaac aaagggtaat atccggaaac ctcctcggat tccattgccc agctatctgt 360

cactttattg tgaagatagt ggaaaaggaa ggtggctcct acaaatgcca tcattgcgat 420

aaaggaaagg ccatcgttga agatgcctct gccgacagtg gtcccaaaga tggacccccа 480

cccacgagga gcatcgtgga aaaagaagac gttccaacca cgtcttcaaa gcaagtggat 540

tgatgtgata tctccactga cgtaagggat gacgcacaat cccactatcc ttcgcaagac 600

ccttcctcta tataaggaag ttcatttcat ttggagagga cacgctgaca agctgactct 660

agcagatcta ccgtcttcgg tacgcgctca ctccgccctc tgcctttgtt actgccacgt 720

ttctctgaat gctctcttgt gtggtgattg ctgagagtgg tttagctgga tctagaatta 780

cactctgaaa tcgtgttctg cctgtgctga ttacttgccg tcctttgtag cagcaaaata 840

tagggacatg gtagtacgaa acgaagatag aacctacaca gcaatacgag aaatgtgtaa 900

tttggtgctt agcggtattt atttaagcac atgttggtgt tatagggcac ttggattcag 960

aagtttgctg ttaatttagg cacaggcttc atactacatg ggtcaatagt atagggattc 1020

atattatagg cgatactata ataatttgtt cgtctgcaga gcttattatt tgccaaaatt 1080

agatattcct attctgtttt tgtt gtgtg ctgttaaatt gttaacgcct gaaggaataa 1140

atataaatga cgaaattttg atgtttatct ctgctccttt attgtgacca taagtcaaga 1200

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tcagatgcac ttgttttaaa tattgttgtc tgaagaaata agtactgaca gtattttgat 1260

gcattgatct gcttgtttgt tgtaacaaaa tttaaaaata aagagtttcc tttttgttgc 1320

tctccttacc tcctgatggt atctagtatc taccaactga cactatattg cttctcttta 1380

catacgtatc ttgctcgatg ccttctccct agtgttgacc agtgttactc acatagtctt 1440

tgctcatttc attgtaatgc agataccaag cggcctctag aggatccagg agcaaccatg 1500

gcgcaagtta gcagaatctg caatggtgtg cagaacccat ctcttatctc caatctctcg 1560

aaatccagtc aacgcaaatc tcccttatcg gtttctctga agacgcagca gcatccacga 1620

gcttatccga tttcgtcgtc gtggggattg aagaagagtg ggatgacgtt aattggctct 1680

gagcttcgtc ctcttaaggt catgtcttct gtttccacgg cgtgc atg gcc gct tgc 1737
                                                Met Ala Ala Cys

gag ctt cgc cca gcc acg cag tac gac acc gac gcc gtg tac gcg ctg   1785
Glu Leu Arg Pro Ala Thr Gln Tyr Asp Thr Asp Ala Val Tyr Ala Leu
  5                  10                  15                  20

atc tgc gag ctc aag cag gcg gag ttc gac cac cac gcc ttc cgc gtg   1833
Ile Cys Glu Leu Lys Gln Ala Glu Phe Asp His His Ala Phe Arg Val
                 25                  30                  35

ggc ttc aac gcc aac ctg cgc gac ccc aac atg cgc tac cat ctg gcg   1881
Gly Phe Asn Ala Asn Leu Arg Asp Pro Asn Met Arg Tyr His Leu Ala
             40                  45                  50

ctg ctt gat ggc gaa gtg gtc ggc atg atc ggc ctg cac ctc cag ttc   1929
Leu Leu Asp Gly Glu Val Val Gly Met Ile Gly Leu His Leu Gln Phe
         55                  60                  65

cac ctg cat cat gtc aac tgg atc ggc gag atc cag gag ctg gtc gtg   1977
His Leu His His Val Asn Trp Ile Gly Glu Ile Gln Glu Leu Val Val
     70                  75                  80

atg cca cag gcg agg ggt ctg aac gtc ggc agc aag ctc ctg gcg tgg   2025
Met Pro Gln Ala Arg Gly Leu Asn Val Gly Ser Lys Leu Leu Ala Trp
 85                  90                  95                 100

gcc gag gag gaa gcc agg cag gcc gga gcc gag atg acc gag ctc agc   2073
Ala Glu Glu Glu Ala Arg Gln Ala Gly Ala Glu Met Thr Glu Leu Ser
                105                 110                 115

acc aac gtg aag cgc cac gac gcg cac cgc ttc tac ctg cgc gaa ggc   2121
Thr Asn Val Lys Arg His Asp Ala His Arg Phe Tyr Leu Arg Glu Gly
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tac gag cag agc cac ttc cgc ttc acc aag gcg ctg taaagatctg        2167
Tyr Glu Gln Ser His Phe Arg Phe Thr Lys Ala Leu
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aattcccgat cgttcaaaca tttggcaata aagtttctta agattgaatc ctgttgccgg 2227
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tcttgcgatg attatcatat aatttctgtt gaattacgtt aagcatgtaa taattaacat 2287

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gtcatctatg ttactagatc ggggatatc 2436

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<212> PRT
<213> Artificial Sequence

<400> 32

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Val	Tyr	Ala	Leu	Ile	Cys	Glu	Leu	Lys	Gln	Ala	Glu	Phe	Asp	His	His
			20					25					30		
Ala	Phe	Arg	Val	Gly	Phe	Asn	Ala	Asn	Leu	Arg	Asp	Pro	Asn	Met	Arg
		35					40					45			
Tyr	His	Leu	Ala	Leu	Leu	Asp	Gly	Glu	Val	Val	Gly	Met	Ile	Gly	Leu
	50					55					60				
His	Leu	Gln	Phe	His	Leu	His	His	Val	Asn	Trp	Ile	Gly	Glu	Ile	Gln
65					70					75					80
Glu	Leu	Val	Val	Met	Pro	Gln	Ala	Arg	Gly	Leu	Asn	Val	Gly	Ser	Lys
				85					90					95	
Leu	Leu	Ala	Trp	Ala	Glu	Glu	Glu	Ala	Arg	Gln	Ala	Gly	Ala	Glu	Met
			100					105					110		
Thr	Glu	Leu	Ser	Thr	Asn	Val	Lys	Arg	His	Asp	Ala	His	Arg	Phe	Tyr
		115					120					125			
Leu	Arg	Glu	Gly	Tyr	Glu	Gln	Ser	His	Phe	Arg	Phe	Thr	Lys	Ala	Leu
	130					135					140				

INTERNATIONAL SEARCH REPORT

Interna .al Application No
PCT/US 99/27152

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/54 C12N9/10 C07K16/40 C12N5/10
C12Q1/68 A01H5/00 A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 26913 A (PLANT GENETIC SYSTEMS NV ;CORNELISSEN MARCUS (BE); REYNAERTS ARLET) 24 November 1994 (1994-11-24) page 4, paragraph 5 -page 5, paragraph 3 example 2 ---	1-20, 40-42
X	WO 97 03205 A (BAYER AG ;HAIN RUEDIGER (DE); FISCHER REGINA (DE)) 30 January 1997 (1997-01-30) page 2, line 4 -page 2, line 13 page 10, line 3 -page 10, line 20; example C --- -/--	1-20, 40-42

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
13 April 2000	27/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Burkhardt, P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	YAKOVLEVA G M ET AL: "Phosphate-independent expression of the carbon-phosphorus lyase activity of Escherichia coli." APPLIED MICROBIOLOGY AND BIOTECHNOLOGY MAY, 1998, vol. 49, no. 5, May 1998 (1998-05), pages 573-578, XP000885947 ISSN: 0175-7598 page 574, column 2, paragraph 3; figure 1; table 1	31-38
X	MAKINO K ET AL: "MOLECULAR ANALYSIS OF THE CRYPTIC AND FUNCTIONAL PHN OPERONS FOR PHOSPHONATE USE IN ESCHERICHIA-COLI K-12" JOURNAL OF BACTERIOLOGY 1991, vol. 173, no. 8, 1991, pages 2665-2672, XP000885441 ISSN: 0021-9193 page 2666, paragraph 3; figure 2; table 1	26-39
X	BURLAND V ET AL: "ANALYSIS OF THE ESCHERICHIA COLI GENOME VI: DNA SEQUENCE OF THE REGION FROM 92.8 THROUGH 100 MINUTES" NUCLEIC ACIDS RESEARCH,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 23, no. 12, 25 June 1995 (1995-06-25), pages 2105-2119, XP000612159 ISSN: 0305-1048 page 2106, paragraph 2; figure 1; table 1	26-39
X	CHEN C -M ET AL: "MOLECULAR BIOLOGY OF CARBON-PHOSPHORUS BOND CLEAVAGE. CLONING AND SEQUENCING OF THE PHN (PSID) GENES INVOLVED IN ALKYLPHOSPHONATE UPTAKE AND C-P LYASE ACTIVITY IN ESCHERICHIA COLI B" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 265, no. 8, 1 March 1990 (1990-03-01), pages 4461-4471, XP000612157 ISSN: 0021-9258 figure 2; table 2	26-39

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BARRY G ET AL: "INHIBITORS OF AMINO ACID BIOSYNTHESIS: STRATEGIES FOR IMPARTING GLYPHOSATE TOLERANCE TO CROP PLANTS" , CURRENT TOPICS IN PLANT PHYSIOLOGY,US,AMERICAN SOCIETY OF PLANT PSYSIOLOGISTS, ROCKVILLE, MD, PAGE(S) 139-145 XP000566269 ISSN: 1057-6576 page 141, paragraph 4 -page 142, last paragraph ---	1-25
A	SAROHA M K ET AL: "GLYPHOSATE-TOLERANT CROPS: GENES AND ENZYMES" JOURNAL OF PLANT BIOCHEMISTRY & BIOTECHNOLOGY, vol. 7, July 1998 (1998-07), pages 65-72, XP000866487 the whole document -----	1-25

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/US 99/27152

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9426913 A	24-11-1994	AU 684785 B	08-01-1998
		AU 6797294 A	12-12-1994
		EP 0698106 A	28-02-1996
		US 5962768 A	05-10-1999
WO 9703205 A	30-01-1997	DE 19525034 A	16-01-1997
		AU 6304896 A	10-02-1997
		EP 0837945 A	29-04-1998
		JP 2000500002 T	11-01-2000

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